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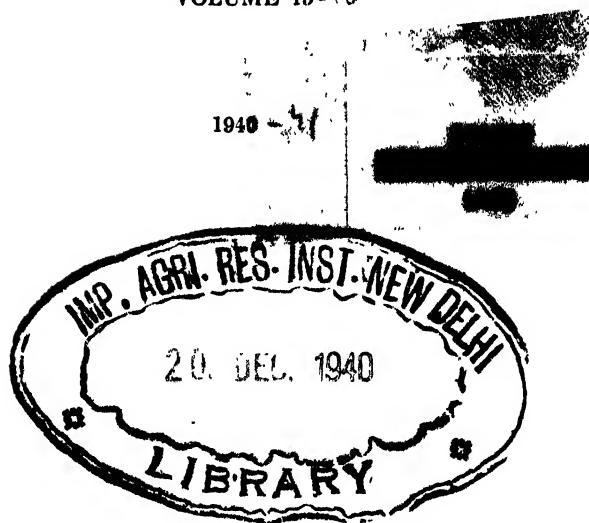
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STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

VOLUME 15-16

1940 - 41



Published by
BIOTECH PUBLICATIONS
GENEVA, N. Y., U. S. A.

Printed in the U. S. A.

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STAIN TECHNOLOGY

VOLUME 15

JANUARY, 1940

NUMBER 1

19812

PROGRESS IN THE STANDARDIZATION OF STAINS

BIOLOGICAL STAINS IN TIME OF WAR

The Biological Stain Commission was conceived as the result of the World War. Now it faces another European war, and it is interesting to notice the differences in the situation.

In 1914 there were no American-made dyes. All stains used in American laboratories were of German manufacture, mostly the product of two concerns, which had originated from the efforts of a single man and were often confused in this country so that the two lines of German stains were thought to be one. When the United States entered the war the various new laboratories which were set up at that time were unable to obtain German dyes and found the American products unreliable. It was to meet this situation and to put the use of stains on a more scientific basis that the Stain Commission was established.

Today, the situation could hardly be more different. Nearly every large nation has its dye industry, and that of the United States is unsurpassed. Thanks to the cooperation of the manufacturers on the one hand and numerous scientists on the other, the production of biological stains is now on a scientific basis; the manufacturers know what biologists want and the biologists know how to make any new requirements they may have intelligible to the industry. Furthermore, practically all stains and every intermediate needed for their manufacture can be obtained from American sources. There probably are *no* exceptions to this statement, altho there are a few of the less common dyes and compound stains which have been largely imported lately because of difficulties that have been obtained with some of the American products. Among these have been brilliant cresyl blue and Giemsa stain. It is interesting, by the way, to note that American stain companies report a greatly increased demand for these two particular stains since September first.

There has, as a matter of fact, been a general stimulation to the stain industry since summer. This has been reflected by the orders the stain companies have sent to the Stain Commission for certification labels. The increased demand began in August, so it could not

have been due wholly to the shutting off of the European sources of supply. September, and then again October, broke all preceding records in the demand for certification labels. This has been very gratifying, especially now that the Commission is no longer being financed by the chemical foundation. If the increased demand proves to be more than temporary, the Commission may become self-supporting. Users of stains can help make it permanent by taking pains to see that their institutions order Commission certified stains.

H. J. CONN

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the October number of this Journal.

STAINS CERTIFIED SEPT. 1, 1939 TO NOV. 30, 1939*

| Name of dye | Certification No. of batch | Dye Content | Objects of tests made by Commission† | Date approved |
|-------------------------|----------------------------|-------------|-----------------------------------------------------------------------------------|----------------|
| Methylene blue chloride | NA-14 | 87% | As histological and bacteriological stain, and as constituent of blood stains | Sept. 8, 1939 |
| Brilliant cresyl blue | NV-17 | 57% | As vital stain for blood | Sept. 30, 1939 |
| Sudan IV | I.Z-2 | 81% | As stain for fat | Oct. 13, 1939 |
| Indigo carmine | II-1 | 83% | As histological stain | Oct. 13, 1939 |
| Fast green FCF | CGf-1 | 90% | As histological and cytological counterstain | Oct. 13, 1939 |
| Methylene blue chloride | DA-4 | 84% | As histological and bacteriological stain, and as constituent of blood stains | Nov. 1, 1939 |
| Basic fuchsin | NF-32 | 98% | For general staining, the Feulgen reaction and in bacteriological media | Nov. 2, 1939 |
| Brilliant cresyl blue | NV-18 | 68% | As vital stain for blood | Nov. 8, 1939 |
| Sudan IV | NZ-11 | 64% | As stain for fat | Nov. 10, 1939 |
| Janus green B | NJ-8 | 73% | As vital stain for blood | Nov. 15, 1939 |
| Crystal violet | MC-1 | 98% | As histological, cytological, bacteriological stain, and in bacteriological media | Nov. 21, 1939 |
| Eosine B. | NEb-8 | 80% | As histological counterstain | Nov. 24, 1939 |
| Methylene azure | LAz-1 | 68% | As histological stain and as constituent of blood stains | Nov. 27, 1939 |

Special note:—Attention is called to the two samples of brilliant cresyl blue certified within about six weeks of each other. Both were submitted by the same company. Such a short interval between samples submitted has never occurred before; it seems to indicate a suddenly increased demand for Commission Certified samples of this particular dye.

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

A STANDARDIZED TECHNIC FOR SAFRANIN O¹

CHARLES H. SAWYER, *Yale University, New Haven, Conn.*

ABSTRACT.—A method for control of staining with Safranin O is described. The procedure is as follows:

Overstain the sections, freed of paraffin, 4 hours or more in 0.1% solutions of either light green S F or fast green F C F in 50% alcohol. These solutions are adjusted to pH 2.4 with 0.1 N HCl. Rinse in distilled water.

Destain at least 30 minutes in Sörensen's Buffer pH 8. Rinse in distilled water.

Overstain in 0.1% Safranin O, 4 hours or more. Rinse in distilled water.

Destain 15 minutes in 0.01 N HCl (pH 2) or in 0.001 N HCl (pH 3) depending on whether light green or fast green, respectively, is the counterstain. The acid solutions are freshly prepared from a stock solution of 0.1 N acid. Rinse in distilled water.

Dehydrate in two changes of dioxan, pass thru xylol and mount in balsam.

Safranin O, one of the most valuable nuclear stains, has always presented difficulties to the histologist; it has given extremely variable results (Conn, 1936, p. 86). At least a part of this inconstancy has been due to the solubility of the stain in alcohol. Destaining and dehydrating are usually performed simultaneously and often too much of the stain washes out before the preparation is mounted. The aim of the present work has been to obviate this phase of the difficulty by controlling the destaining process in an aqueous solution of definite pH and by dehydrating in dioxan (Guyer, 1936, p. 64). The author is grateful to Dr. Petrunkevitch, who devised the method (Petrunkevitch, 1937), and under whose supervision the work was done.

It was first necessary to find the optimum pH to use for destaining. A curve (Fig. 1) was prepared to enable one to make up quickly a series of 0.1% aqueous solutions of the stain from pH 2 to pH 10. The procedure was to add varying amounts of 0.1 N HCl or NaOH to 25 ml. samples of a stock solution of 0.2% Grübler's Safranin O and to dilute with distilled water to 50 ml. in a volumetric flask. A Beckman glass-electrode pH-meter was used in pH measurements,

¹Contribution from the Osborn Zoological Laboratory, Yale University.

and all the readings were taken at 23° C. From the curve a stain series was set up ranging from pH 2 to pH 9.

Since staining affinity varies widely with the fixation employed, four representative fixing fluids were used: Petrunkevitch's paranitrophenol-cupric-nitrate-nitric (Guyer, p. 34), Zenker's bichromate-sublimate-sodium-sulphate-acetic (Lee, 1937, p. 46), Bouin's micro-

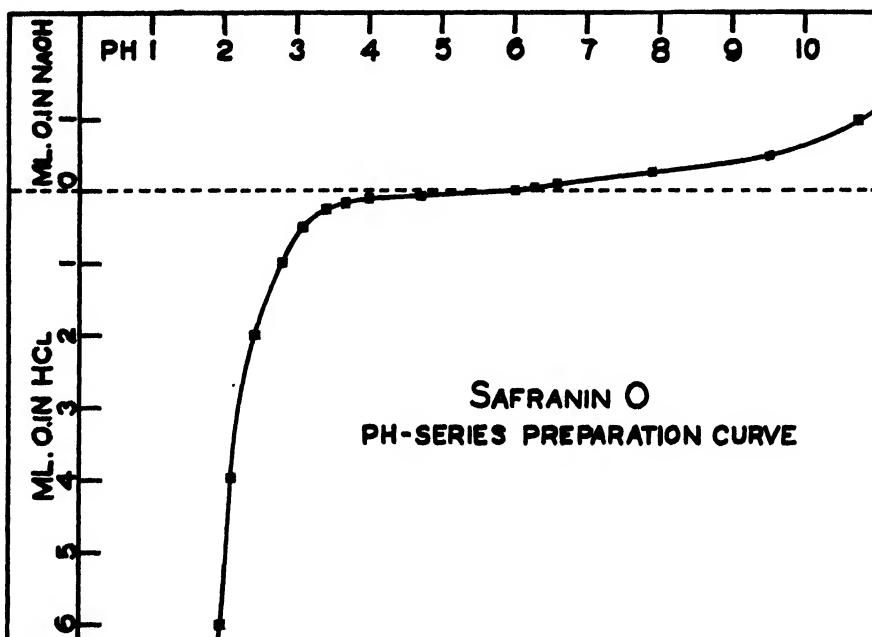


Fig. 1. Curve showing amount of 0.1 N acid or alkali needed to make up 50 ml. 0.1% Safranin O at various pH-values.

formol-acetic (Lee, p. 58), and Petrunkevitch's sublimate-nitric-acetic-alcohol (Lee, p. 45). The tissue, intestine of the leopard frog, was fixed in these fluids and sectioned at 10 μ in paraffin. The sections, freed of paraffin, were stained in the pH-series overnight to insure completion of the staining reaction. Each was then rinsed in an HCl or NaOH solution of approximately the same H-ion concentration as the stain, dehydrated in two changes of dioxan, passed thru xylol, and mounted in balsam. Treatment preliminary to staining may be with either alcohol or dioxan, but the latter is quicker.

From the results (Figs. 2-5) it can be seen that the greatest differentiation between nuclear and cytoplasmic staining is in each case at pH 3 or 4 and that nearly maximal staining occurs at pH 6, the stain prepared with no addition of acid or alkali. At pH 2 the material fixed in Bouin's or Zenker's fluids shows no nucleo-cytoplasmic differentiation; maximal differentiation is reached at pH 4. The Bouin-

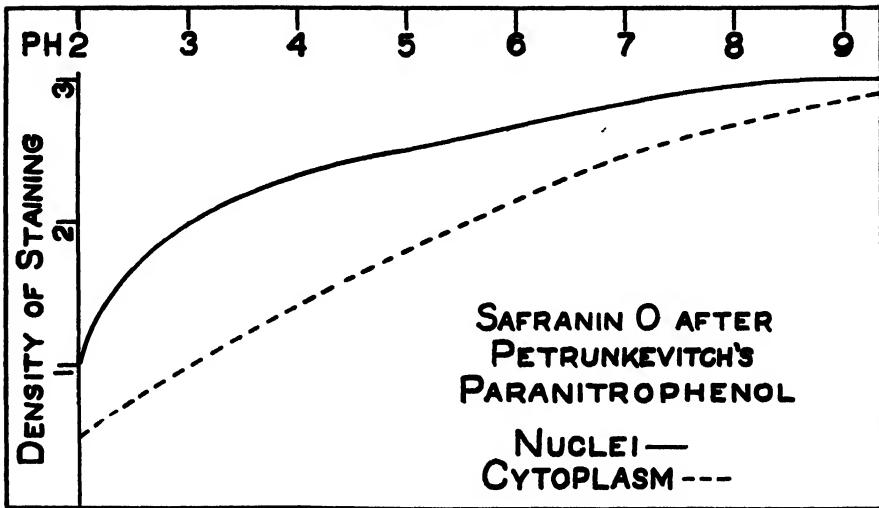


Figure 2.

fixed sections were unusual in that the secretion-filled goblet cells of the lining epithelium had a stronger affinity for the stain than did the nuclei—taking the stain even at a reaction of pH 2. The tissue fixed in Petrunkevitch's fluids showed its highest differentiation at pH 3. No color comparator was used in judging differentiation, but the results were sufficiently clear-cut so that the estimations of relative density designated on the curves as 1, 2, and 3 for slight, medium and maximum, are fairly accurate.

On the basis of these results the destaining method was attempted, keeping in mind the rule that the capacity of retaining a stain in a de-

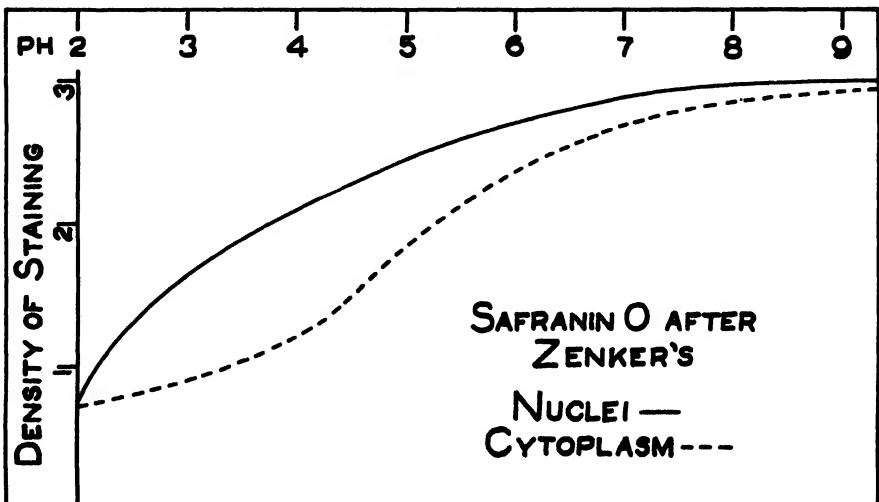


Figure 3.

staining fluid of a given pH-value is not the same as the staining affinity at that pH-value. Inasmuch as the stain without treatment by acid or alkali gave nearly maximal affinity, it was used untreated for the preliminary overstaining. Destaining at pH 2 (0.01 N HCl, prepared by diluting 5 ml. 0.1 N HCl to 50 ml. in a volumetric flask) gave the following results: Zenker-fixed material, no differentiation; Bouin-fixed, goblet cells only; Petrunkevitch's sublimate or *p*-nitrophenol-fixed, excellent differentiation, only the chromatin retaining

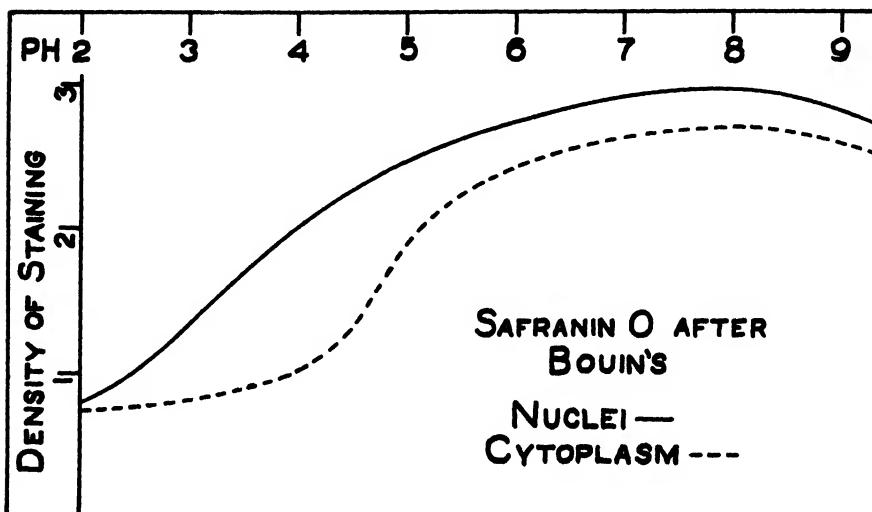


Figure 4.

the stain. Optimum destaining for Bouin- or Zenker-fixed tissue was achieved at pH 3, but the Bouin-fixed material never gave excellent differentiation. Of the Petrunkevitch fixatives the *p*-nitrophenol gave slightly the more striking contrast. Tho the destaining process is theoretically independent of the time element, provided completion of the destaining reaction is reached, prolonged exposure to pH 2 tends to make the sections slip off the slide; 15 minutes is sufficient for optimal destaining in acid.

Two green counterstains were successfully employed, light green S F (Coleman and Bell Co.) and fast green F C F (National Aniline and Chemical Co.). These gave optimum results when the sections were overstained in 0.1% solution in 50% alcohol at pH 2.4 and de-stained at pH 8 with Sörensen's phosphate buffer. Each of the acid staining solutions was made up by taking 10 ml. of 0.5% aqueous solution of the stain, adding 25 ml. of absolute alcohol and 4 ml. of 0.1 N HCl, and diluting with water to 50 ml. Best results were obtained by applying the counterstain before staining with Safranin O.

Of the two greens, light and fast, the latter is much the better from the point of view of permanency. Safranin O, however, has a weaker affinity for sections previously counterstained by fast green; so if the latter is used the final destaining is better effected at pH 3 (0.001 N HCl).

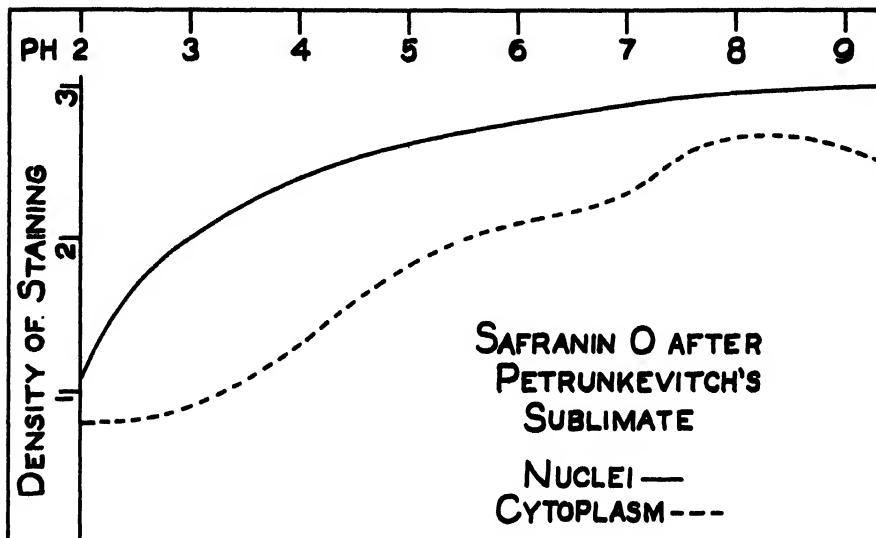


Figure 5.

Not all stains are amenable to the treatment described here. It may appropriately be reported that crystal violet gives negative results on application of this technic. One can tell only by experiment whether or not a particular stain will react favorably to the treatment. As has been stated, the procedure for Safranin O was developed using a sample of Grubler's ("water soluble"). The method has since been satisfactorily applied to a second batch of Grubler's and to a sample from the National Aniline and Chemical Co. (Certification No. NS-9, dye content 97%), so it should be generally applicable to Safranin O.

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STAINING PARAFFIN SECTIONS WITH PROTARGOL

5. CHLORAL HYDRATE MIXTURES, WITH AND WITHOUT FORMAMIDE, FOR FIXING PERIPHERAL NERVES.¹

EDWARD W. BANK² and H. A. DAVENPORT, *Department of Anatomy, Northwestern University, Chicago, Ill.*

ABSTRACT.—A series of experiments was directed toward finding a means of improving fixation of mammalian glandular tissue and peripheral nerves with chloral hydrate. Specimens from cat, dog, rat, guinea pig, and man were fixed in solutions of 5–15% chloral hydrate in ethyl, methyl, and propyl alcohols, both pure and diluted with varying amounts of water. Modifiers were added, including acids, alkalies, alkaloids, amines, formamide, pyridine, piperidine, and formalin. The sectioned material was stained by the 2-hour method (AgNO_3 -protargol) described previously (Davenport et al., 1939). The acidification of alcoholic chloral hydrate mixtures was deleterious to fixation but alkalinization was not. Among the modifiers, formamide was the one which showed definite improvement of fixation. A 10% solution of formamide alone in 50% ethyl alcohol gave good fixation and staining of peripheral nerve trunks, but addition of 5–7% chloral hydrate to this mixture improved staining. Treatment with 1% ammoniated alcohol after fixation and before embedding was of no value in section staining. Block stains were not tried.

In the preceding paper of this series (Davenport, McArthur, and Bruesch, 1939) a two-hour method for staining nervous tissue was described. The method was worked out chiefly on spinal nerve roots, tissue from the central nervous system, and sympathetic trunks. The use of acid mixtures recommended for fixing central nervous tissue and roots has not proved very satisfactory for peripheral nerve trunks and is particularly bad for glandular tissue. The present study has been directed toward improvement in fixation and differentiation of the more peripherally located nerves by the use of neutral or alkaline fixatives based on mixtures of chloral hydrate, alcohol and water. Chloral hydrate was chosen because it has been

¹Contribution No. 903 from the Department of Anatomy, Northwestern University Medical School.

²Submitted by E. W. Bank as a partial requirement for the degree, Master of Science, to the Graduate School of Northwestern University.

TABLE 1. LIST OF FIXATIVES*

| No. | Chloral hydrate | Modifier | Alcohol | Water |
|-------|-----------------|--------------------------------|---------------------------|----------------------------|
| 1 | 15 | 0 | methyl celo-solve, Id. | 100 70 |
| 2 | 15 | 0 | n-propyl, abs. ethyl, | 70 30 30 0 |
| 3 | 15 | 0 | " " | 70 |
| 4 | 15 | 0 | " " | 70 |
| 5 | 15 | 0 | " " | 30 |
| 6 | 15 | 0 | " " | 30 |
| 7 | 15 | 0 | " " | 70 |
| 8 | 15 | 0 | methyl, | 100 0 |
| 9 | 15 | 0 | " " | 70 30 |
| 10 | 15 | 0 | " " | 30 70 |
| 11 | 12 | amm. w., 0.5† | n-propyl, " " | 60 60 |
| 12 | 12 | quinine hydrochloride, 2 | " " | 40 |
| 13 | 12 | glacial acetic, 5 | " " | 40 |
| 14 | 12 | monochloracetic, 0.2 | " " | 40 |
| 15 | 12 | " 0.2 | ethyl | 60 40 |
| 16 | 12 | 0 | pyridine, | 50 50 |
| 17 | 12 | amm. w., 0.25 | propyl, ethyl | 50 5 45 |
| 18 | 12 | piperidine, 0.25 | propyl, ethyl | 50 5 45 |
| 19 | 12 | n-butylamine, 0.25 | propyl, ethyl | 50 7.5 42.5 |
| 20 | 12 | formamide, 5 | propyl, ethyl | 50 5 45 |
| 21 | 12 | ethanolamine, 3.5 | propyl, ethyl | 50 5 45 |
| 22 | 12 | 0 | propyl, ethyl | 50 15 35 |
| 23 | 7 | formamide, 15 | propyl, | 30 50 |
| 24 | 7 | amm. w., 0.25 | butyl, | 15 5 60 |
| 25 | 0 | formamide, 20 | ethyl | 15 5 60 |
| 26 | 0 | formamide, 20 | butyl, | 15 py, 5 60 |
| 27 | 0 | formamide, 20 | 0 0 80 | 78 |
| 28 | 0 | formamide, 20 | 0 0 80 | 50 |
| 29 | 5 | formalin, 2 | 0 0 0 0 0 | 50 50 50 50 50 |
| 30 | 5 | brucine, 5 | n-propyl, " " | 50 50 |
| 31 | 5 | " 5 | methyl, | 100 0 |
| 32** | 5 | " 5 | ethyl, | 40 40 |
| 33*** | 5 | Py, 20 | ethyl, | 50 50 |
| 34† | 3 | amm. w., 0.2 | ethyl, | 50 50 |
| 35 | 7 | formalin, 12 | ethyl, | 50 pH 50 |
| 36 | 7 | formamide, 7 | propyl, | 35-4.9 35-8.9 50 |
| 37 | 7 | " 7+amm. w., 1.0 | " | 35-7.4 50 |
| 38 | 7 | " 7 " 0.1 | " | 35-4.7 50 |
| | | " 7+trichlor-acetic acid, 0.01 | " | 35-4.7 50 |

*Formamide, piperidine, n-butylamine, ethanolamine, and n-propyl alcohol were obtained from Eastman Kodak Co., Rochester, N. Y. Protargol was obtained from Winthrop Chemical Co., New York, N. Y.

**d'Ancona's fluid No. 1

***d'Ancona's fluid No. 2

†One of Cajal's formulae.

Abbreviations: amm. w., strong ammonia water; Py, pure pyridine (Merck).

TABLE 1. LIST OF FIXATIVES (continued)

| No. | Chloral hydrate | Modifier | Alcohol | Water |
|-----|-----------------|------------------------------------------|--------------|-------|
| 39 | 7 | formamide, 7 + trichlor-acetic acid, 0.1 | " 35-4.1 | 50 |
| 40 | 0 | formamide, 7 | " 35-4.9 | 50 |
| 41 | 7 | " 7 | ethyl 35-4.9 | 50 |
| 42 | 0 | " 7 | " 35-5.0 | 50 |
| 43 | 7 | " 0 | " 50 | 50 |
| 44 | 0 | formamide, 10 | " 50 | 50 |
| 45 | 7 | " 10 | " 50 | 50 |
| 46 | 7 | " 10 + Py, 15§ | " 35 | 50 |
| 47 | 7 | " 10 + Py, 15+ | " 35 | 50 |
| | | formalin, 4 | " 0 | 100 |
| 48 | 7 | formamide, 10 | methyl, 50 | 50 |
| 49 | 7 | " 10 | propyl, 50 | 50 |
| 50 | 7 | " 10 | ethyl, 50 | 50 |
| 51 | 0 | 0 | propyl, 50 | 50 |
| 52 | 0 | 0 | propyl, 50 | 50 |

§Py = pure pyridine (Merck).

used successfully by previous investigators (Cajal 1907, 1910, 1929; d'Ancona 1925; and Perez 1932) for nerve endings when block staining of the Cajal type was used.

Material and Methods. Nearly all the experiments were made by using 6-10 fixatives in a group and fixing in each of these a piece of nerve and two pieces of glandular tissue, such as pancreas, thyroid, adrenal and salivary glands, from the same animal. Represented in the series were 7 cats, 4 dogs, 6 rats, 1 guinea pig, and 1 human. The tissues were fixed by immersion, and the time was about 20 hours. Embedding in paraffin, cutting, and mounting the sections on the slide were done in the routine manner. Paraffin was removed and the slides passed to water thru graded alcohol, impregnated 1 hour at 60° C. with 5% aqueous AgNO₃ solution, washed in 3 changes of distilled water 0.5 minutes each and put into 0.2% protargol solution at 27° C. for 1 hour. Following impregnation a rinse of 1 or 2 seconds in distilled water was given and the protargol reduced by amidol-sulfite solution (amidol, 0.1 g.; NaHSO₃, 1.0 g.; Na₂SO₃, 10 g.; and water, 100 cc.) for 1-2 minutes. The reducing solution was made up immediately before using by adding the dry amidol to a stock solution of the sulfites. The slides were washed carefully to remove all reducing solution and toned in a 0.1% solution of AuCl₃ for about 5 minutes, washed again and, if the stain were light, reduced 0.5 minutes with a 0.5% amidol solution; if dark, reduced 5 minutes with a mixture of oxalic acid and formalin (oxalic acid, 2 g.; formalin, 1 cc.; water 100 cc.). To insure the removal of unreduced AgCl, a

5-10% "hypo" solution should be used after the second reduction, but this was not done in a routine way, because the preparations were kept only a short time. A final washing followed by dehydration and covering in balsam completed the staining. The procedure has a few minor modifications of the original method.

Fixatives: The 52 fixatives listed in Table 1 are about half of the total number tried, but include those which are characteristic representatives of the various modifiers used. We had in mind the following questions:

1. Is there any advantage of methyl or *n*-propyl alcohol over ethyl?
2. What is the proper water content of these alcohols when used with chloral hydrate?
3. What is the effect of adding acid or base?
4. Does the addition of amines, amine-like substances, or alkaloids improve fixation or subsequent staining with silver?

In addition to the fixatives in Table 1, 12% chloral hydrate solutions in isopropyl, allyl, *n*-butyl, isobutyl, and octyl alcohols and in dioxan were tried. With the exception of isopropyl alcohol, excessive shrinkage occurred with their use. Our findings with regard to the higher alcohols agree with Cajal's (1907) observations on their lack of utility in similar fixing solutions for staining in the block.

In one series of tissue samples (fixatives 43-51) the specimens were cut in two after fixation and one half given a further treatment of 24 hours in 1% ammoniated alcohol. Such treatment seemed to be of no value or even deleterious to both staining and fixation, as shown by paler stains and greater shrinkage.

Methyl cellosolve and dioxan were poor substitutes for alcohol.

Results and Discussion: No advantage of methyl or propyl alcohol over ethanol appeared consistently. When used without dilution with water, propyl alcohol caused more generalized shrinkage than either methanol or ethanol. It was found that 10-15% solutions of chloral hydrate in mixtures of water and propyl alcohol separated into two layers when the water content exceeded 40%, but these could be made into perfect solutions by the addition of a small amount of ethyl alcohol, ethanolamine, formamide, ammonia or other modifiers used in fixatives 17 to 22. Ethyl and methyl alcohols gave perfect solutions when mixed with any amount of water.

The answer to question 2, regarding water content of the fixative, appears to be that only methyl alcohol can be used pure with impunity as a solvent for chloral hydrate in fixation. Ethyl and propyl alcohols cause excessive shrinkage when so used. Ethanol as 50 and 70% mixtures with water, and propanol diluted to about 40% with

sufficient formamide (fixative 35) to homogenize the solution were definitely more satisfactory than higher concentrations.

The addition of acids was deleterious, but the addition of ammonia, piperidine, pyridine, and butylamine in small amounts was not. Addition of acids, except in relatively minute amounts (fixative 38), caused serious cracking of the tissue, especially peripheral nerves, with the result that cross sections presented a mosaic-like pattern caused by longitudinal fissures—a type of artifact commonly seen after fixation in neutral formalin. In some specimens the addition of alkali favored the subsequent differentiation of nerve fibers in glands, but in others this effect was not seen. It appeared to have no effect on fixation *per se*.

Of all the modifiers tried, only one showed a consistently beneficial effect both on fixation and on subsequent staining. This was formamide, and while it was used by Cajal (1907) as a 4% solution in 96% alcohol, we have found no mention of its use in conjunction with chloral hydrate. This substance, altho it has a chemical formula like an amine, has properties more nearly resembling urea in that it is able to denature protein and is not alkaline. As an addition to chloral hydrate solutions, it seems to be well worth while. Used alone (without chloral hydrate) in water-ethanol or water-propanol mixtures (fixatives 40 and 44) it has given superior fixation and strong staining with silver. The addition of chloral hydrate to the mixture, however, appears to give some improvement in the differentiation of nerve fibers.

The use of formalin (fixatives 28, 34, and 47) was deleterious both to fixation and staining.

We wish to suggest that fixatives having formulas the same as, or similar to, those given in fixatives 23, 24, 35, 36, 41, 45, 49 and 50 are worthy of trial in the staining of peripheral nerve trunks, particularly when enumeration of fibers is desired.

Results with glandular material have been only partially successful; hence the use of chloral hydrate mixtures, altho a great improvement over acid fixatives, leaves much to be desired in section staining with the silver-nitrate-protargol method.

We wish to acknowledge aid from the National Youth Administration to E. W. Bank.

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A PROCEDURE FOR STAINING FILAMENTOUS ALGAE AND FUNGI ON THE SLIDE

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The Venetian Turpentine method for mounting delicate structures such as filamentous algae requires so much handling of the bulk material that tangling, twisting, and excessive breaking of the filaments is almost unavoidable. The conjugating filaments of forms which show scalariform conjugation are likely to be separated and otherwise distorted. Due to matting of the filaments, uniform destaining is difficult of accomplishment, and satisfactory teasing-out of the matted filaments in the thick Venetian Turpentine just prior to covering is difficult or impossible.

The following procedure, based upon the use of a modification of Haupt's¹ gelatine fixative, has been used successfully by the writer in overcoming these difficulties and in shortening the preparation time.

Dissolve 1.5 g. of a good grade of gelatin in 100 ml. of distilled water at 30–35° C. Add 5 ml. of glycerin and 2 g. of phenol. Smear a thin film of the fixative upon a warm slide.

After killing and washing the bulk material in running water remove enough for a slide with fine forceps. Place this upon the smeared slide with a "dragging" movement, beginning at one end of the slide and pulling the filaments toward the other end. The water which adheres to the filaments is sufficient for floating them into position; a large quantity of water such as is used for floating paraffin ribbons is unnecessary and undesirable. Most of the filaments will thus arrange themselves in a free and parallel fashion. Conjugating filaments can be mounted in this manner without damage. If desired, long ends extending beyond the area to be covered may be turned in beside the main mass of filaments. Invert the slide over a shallow dish containing a small quantity of commercial (40%) formaldehyde and cover with an inverted dish or low bell jar. Glass staining dishes of the low, rectangular form, slightly less than 3 inches wide, serve this purpose well. Leave the slides thus exposed to the fumes of formaldehyde for $\frac{1}{2}$ hour. Wash slides in distilled water, 5–10 minutes. They are now ready for staining in aqueous stain or for the alcohol series if an alcoholic stain is to be used. Clear in xylene and mount in balsam.

¹Haupt, A. W. 1930. A gelatine fixative for paraffin sections. *Stain Techn.* 5, 97–8.

The following schedule has given good preparations of *Spirogyra*:

1. Kill and fix in chromo-acetic acid (1% chromic acid, 3% glacial acetic acid), 12 hours.
2. Wash in running water, 12 hours.
3. Fix material to slide as described above. Wash in distilled water, 5-10 minutes. (Slides may at this point be run up to 85% alcohol for hardening and returned to water.)
4. Mordant in 2% aqueous solution of ferric ammonium sulfate, 4 hours. Wash in several changes of water, $\frac{1}{2}$ hour.
5. Stain in 0.5% aqueous hematoxylin, 12 hours. Wash in water (2 or 3 changes), $\frac{1}{2}$ hour.
6. Destain with 1% aqueous solution of ferric ammonium sulfate, about 1 hour. Wash in water, $\frac{1}{2}$ hour.
7. Dehydrate by closely graded series of alcohols to 95%.
8. Counterstain with 0.5% orange G in 95% alcohol, 15-30 seconds.
9. Complete dehydration in absolute alcohol, 1-2 minutes. Clear in xylene. Mount in balsam.

Other filamentous algae with this and other combinations of stains have given good preparations. Miss Alma Whiffen of this laboratory has used successfully the present technic for mounting and staining cultures of *Achlya* grown on hemp seed.

FURTHER EXPERIMENTS WITH THE MASSON TRICROME MODIFICATION OF MALLORY'S CONNECTIVE TISSUE STAIN¹

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ABSTRACT.—Several dyes, notably ponceau 2R, azofuchsin 3B, nitrazine yellow, and Biebrich scarlet may replace imported "ponceau de xylidine" in the Masson ponceau acid fuchsin mixture. Of these Biebrich scarlet appears to be the best and may be used without acid fuchsin.

A mixture of equal parts of 5% solutions of phosphomolybdic and phosphotungstic acids is much superior to either acid alone and gives adequate mordanting in 1 minute at 22° C.

With the fast green modification, times in plasma and fiber stains can be reduced to 2 minutes each. With anilin blue a 4-minute plasma stain is required. One-minute final differentiation in 1% acetic acid is adequate.

Primary mordanting of formalin material may be accomplished by 5 minutes in saturated aqueous mercuric chloride or 2 minutes in saturated alcoholic picric acid. Three minutes washing in running water is required after these mordants.

While our previous studies (Lillie, 1938) and those of Goldner (1938) indicated that the trichrome method could be adapted to formalin material and considerably shortened, the length of time (nearly an hour) and the necessity for incubation required by the best of our modifications were still a bar to the frequent or routine use of the method. Moreover, the necessity for an imported "ponceau de xylidene" for the proper execution of the method gave rise to some difficulties in prompt procurement of the dye. Consequently, we determined to test a number of other dyes as to their availability for plasma stains in place of "ponceau de xylidene," and to endeavor to further shorten the procedure.

The following basic technic was used in experiment 9.² Bring paraffin sections of formalin fixed tissues to water as usual. (1) Treat in mordant No. 1 (saturated aqueous picric acid) 10 minutes at 58° C. (2) Wash 15 minutes in running water. (3) Apply Wei-

¹Contribution from Division of Pathology, National Institute of Health.

²Experiments 1 to 8 are included in the previous report (1).

gert's acid iron chloride hematoxylin, 6 minutes. (4) Rinse in water. (5) Stain 5 minutes in plasma stain. (6) Rinse in distilled water. (7) Apply mordant No. 2 (1% phosphomolybdic acid) 10 minutes at 58° C. (8) Stain in 2.5% fast green FCF in 2.5% acetic acid, 5 minutes. (9) Differentiate in 1% acetic acid for 2 minutes. (10) Carry thru alcohol, acetone, acetone+xylene, and xylene to salicylic balsam. The plasma stains were made by mixing 9 cc. of 1% solution of the various dyes with 1 cc. of 1% acid fuchsin and 0.1 cc. glacial acetic acid.

The best plasma stains were given by ponceau 2R, nitrazine yellow, Biebrich scarlet and azofuchsin 3B. Almost as good were Bordeaux red, chromotrope 2R, azofuchsin G, orange G and crocein. Still quite good were "ponceau de xylidene," azofuchsin (C.I. 30), azofuchsin 4G, eosin Y and chrysoidin.³ Distinctly inferior were azofuchsin B, azofuchsin 6B (C.I. 57), azofuchsin 6B or GN (C.I. 154), methyl eosin and erythrosin. Phloxine gave the poorest effect of any. (The sources of these dyes are given in Table 1.) Appendix, pneumonic lung and a nephrosclerotic kidney were used as test objects.

Having noted the statement in Thorpe and Linstead (1933) "that the lakes of complex acids containing both tungsten and molybdenum were much faster than those containing either metal alone" (English patent 216486, 1923), it was decided to compare a mixture of phosphotungstic and phosphomolybdic acids as second mordant with the two acids used alone. In experiment 10 the usual technic was followed, employing Biebrich scarlet with acid fuchsin as the plasma stain. Second mordants were as follows: 1% phosphomolybdic acid, 10 minutes at 58° C.; 1% phosphotungstic acid, 10 minutes at 58° C.; and a mixture of equal parts of 1% solutions of phosphomolybdic and phosphotungstic acids for 5 and 10 minutes at 58° C. and for 5, 10, 15, 30 and 60 minutes at room temperature.

Phosphotungstic acid alone gave distinctly inferior results, but the mixture was excellent at 58° C. for either 5 or 10 minutes and at room temperature when the time was 10 or more minutes.

As Goldner had found he could shorten the phosphomolybdic mordanting time by using more concentrated solutions at room temperature, we decided to try in experiment 11 the effect of raising the concentration of the mixed acids to 5%. The Biebrich scarlet variant was used as before. A control of 1% phosphomolybdic and phosphotungstic acids at room temperature for 10 minutes was used,

³This dye was labeled chrysoidin Y, but was shown not to agree with the characteristics of that dye.

and trials were made of a mixture of equal parts of 5% solutions of the two acids at room temperature for 1, 2, 3, 5, and 10 minutes. All tests gave fully satisfactory results.

In experiment 12 trial was made to see whether the time in the plasma stain could be reduced and whether acid fuchsin could be omitted from the Biebrich scarlet modification. Mordanting was carried on for 1 minute at room temperature with the 5% phosphomolybdotungstic mixture. Plasma stains were applied 1, 2, 3 and 5 minutes, using 1% Biebrich scarlet in 1% acetic acid alone as well as the mixture of Biebrich scarlet and acid fuchsin (9:1).

TABLE I. DYES USED

| Name | C.I. No. | Schultz No. | Manufacturer | Lot No. |
|---------------------------|-------------------------------|-------------|------------------------|------------|
| "Ponceau de xylidene" | | | Grubler | 1.37 |
| Ponceau 2R | 79 | | National Anilin | 7895 |
| Bordeaux red | 88 | 112 | National Anilin | 4988 |
| Chromotrope 2R | 29 | | National Anilin | 8449 |
| Crocein | 252 or 277? | | Old sample | |
| Biebrich scarlet WS | 280 | 247 | Biosol Products | 131 |
| Azofuchsin | 30 | | National Anilin | 7788 |
| Azofuchsin B | 66 | | National Anilin | 3210 |
| *Azofuchsin 4G | 29 * | | National Anilin | 8449 |
| Azofuchsin 6B | 57 | | National Anilin | 3210 |
| Azofuchsin 6B, GN or S | 154 | | National Anilin | 3210 |
| Azofuchsin G | 153 | | Hartman Leddon | (1938) |
| Azofuchsin 3B | 54 | | Hartman Leddon | (1938) |
| Eosin Y | 768 | | National Anilin | Cert. NE11 |
| Methyl eosin | 769? | | Grubler | Old |
| Erythrosin | 773? | | Grubler | Old |
| Phloxine (73%) | 774 | | Hartman Leddon | Recent |
| Acid fuchsin (60%) | 692 | 524 | Hartman Leddon | Recent |
| Methyl blue | 706 | | National Anilin | 7024 |
| Orange G | 27 | | Grubler | 12.37 |
| Nitrazine yellow | | | (Squibbs)† | Recent |
| Chrysoidin (acid dye) | Not C.I. No. 20, 21, or 60 | | Providence Chemical | About 1930 |
| Fast green FCF | | | Coleman and Bell | Recent |
| Anilin blue WS | 707 | | Hartman Leddon | Recent |

*This is listed in the Colour Index as similar to chromotrope 2R, C.I. No. 29.

†The lot actually used was obtained from another source and under a different name; but the company furnishing it to the writer does not put it on the market any more. A sample subsequently obtained from Squibbs labelled "nitrazine" was found to be less concentrated and had to be employed in 2% instead of 1% solution. In this paper the name "nitrazine yellow" is employed for the dye, as it was first described under that name (Wenker, 1934).

Immersion for 2 minutes in plasma stain was found fully adequate, one minute not quite enough. We considered Biebrich scarlet alone to give a better picture than the acid fuchsin mixture.

In experiment 13 trial was made to see whether the time in the

fast green fiber stain and in the acetic acid differentiation could be reduced. The timing was as follows: 2 minutes in Biebrich scarlet (alone), 1 minute at room temperature in the 5% phosphomolybdo-tungstic mixture and 1, 2, 3 and 5 minutes, respectively in the fiber stain with the following 1% acetic differentiation applied 1 and 2 minutes for each variant of the fiber stain. Staining for two minutes in the acetic fast green was adequate, one minute not quite adequate, and one minute differentiation in 1% acetic acid was enough.

Experiment 14 was designed to test the possibility of further reducing the mordanting time in the first mordant. Both saturated aqueous picric acid and saturated aqueous mercuric chloride, which had previously given excellent results as a mordant (Lillie, 1938), were tried at 58° C. for 2, 3, 5 and 10 minutes. The remaining steps were as in experiment 13, with 2 minutes in fast green and 1 minute in 1% acetic acid. The results were essentially identical throughout, indicating that 2 minutes at 58° C. in either of these primary mordants would be adequate. In experiment 15 the same 2 first mordants were used for 2 minutes at 58° C., and trial was made to reduce the washing interval. The balance of the procedure was as in experiment 14. Sections were washed after the first mordants for 1, 2, 3, 5, 10 or 15 minutes or briefly rinsed. A 3-minute washing was required to remove all of the visible yellow color after picric acid mordanting. No difference, however, was seen in the staining with either mordant, regardless of the length of washing.

In experiment 16 the 3-minute washing interval was adopted, the rest of the procedure remaining as before, and trial was made of saturated alcoholic picric acid (about 6%, as compared with about 1.25% for aqueous), saturated aqueous mercuric chloride and 20% alcoholic mercuric chloride as first mordants. These were tried severally for 1, 2, 3, 5 and 10 minutes at room temperature in comparison with saturated aqueous picric acid and mercuric chloride solutions for 2 minutes at 58° C. At room temperature aqueous mercuric chloride gave excellent results at 5 and 10 minutes, good at 3 minutes and irregular with poor staining of muscle at 1 and 2 minutes. Alcoholic mercuric chloride dried on the slides during the brief interval of transfer to running water and results of subsequent staining were spotty and irregular. Mordanting for 1, 3 and 10 minutes gave good results, but for 2 and 5 minutes poor, with muscle almost unstained. Saturated alcoholic picric acid gave satisfactory staining results at all mordanting durations.

In experiment 17 an attempt was made to introduce another color to render the plasma staining more differential. Instead of mixing

the dyes, it was decided to try sequence staining, with or without an intervening mordant. Two dyes were used, orange G and nitrazine yellow, a brown plasma stain, in addition to the Biebrich scarlet. 1% solutions of each were made in 1% acetic acid. Tests were made with each of these dyes, staining for 2 minutes before or after a 2-minute Biebrich scarlet stain, with or without an intervening 1-minute mordanting in 5% phosphomolybdotungstic mixture. When no intervening mordant was used, the effect obtained was the same as from Biebrich scarlet alone. When the intervening mordant was used the effect was that of the first dye alone, whether Biebrich scarlet, orange G or nitrazine yellow without trace of the color expected from the second dye. The procedure was otherwise that of experiment 16 with mordanting for 1 minute in saturated alcoholic picric acid.

Continued use showed some irregularity in plasma staining with 1 minute in the first mordant, so it seemed advisable to increase this to 2 minutes. Thus the modified procedure arrived at from the foregoing experiments is as follows:

1. Bring paraffin sections thru xylol and alcohol into saturated alcoholic picric acid for 2 minutes.
2. Wash 3 minutes in running water.
3. Stain 6 minutes in Weigert's acid iron chloride hematoxylin.
4. Rinse in water.
5. Stain 2 minutes in 1% Biebrich scarlet in 1% aqueous acetic acid.
6. Rinse in water.
7. Mordant 1 minute in a mixture of equal parts of 5% solutions of phosphomolybdic and phosphotungstic acids.
8. Stain 2 minutes in 2.5% fast green FCF in 2.5% aqueous acetic acid.
9. Differentiate 1 minute in 1% aqueous acetic acid.
10. Carry thru alcohol, acetone, acetone+xylene, and xylene (two changes) into salicylic acid balsam.

Longer staining (e.g. 3 or 4 minutes) with Biebrich scarlet is necessary when the following fiber stain is anilin blue or methyl blue.

CONCLUSIONS

1. A number of dyes may be substituted for imported "ponceau de xylidene" in Masson's ponceau acid fuchsin mixture. Among the best are ponceau 2R (C.I. No. 79), azofuchsin 3B (C.I. No. 54), nitrazine yellow, and Biebrich scarlet (C.I. No. 280). We prefer the last to any dye tried thus far. Nitrazine yellow is very good when a

brown plasma stain is desired. Biebrich scarlet makes as satisfactory a plasma stain without addition of acid fuchsin.

2. A mixture of equal parts of phosphomolybdic and phosphotungstic acid solutions is much superior, as a second mordant, to either used alone. When 5% solutions are used, the time required is 1 minute at room temperature.

3. With the fast green modification, the times in the plasma and fiber stains can be reduced to 2 minutes each. With anilin blue a 4-minute plasma stain seems indicated. One-minute differentiation in 1% acetic acid after the fiber stain appears to be adequate.

4. Primary mordanting may be reduced to 5 minutes in saturated aqueous mercuric chloride solution or to 2 minutes in a saturated alcoholic solution of picric acid, both at room temperature. Washing 3 minutes in running water after the mordant seems to be adequate.

The writer is indebted to Scientific Aide Milton Gusack for technical assistance in carrying out the experimental work herein reported.

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THE USE OF SUDAN BLACK B AS A BACTERIAL FAT STAIN

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ABSTRACT.—Sudan black B was introduced as a specific fat stain for the detection of lipids in tissue sections by L. Lison in 1934. Saturated solutions of Sudan black B in 70% alcohol or in ethylene glycol stain the fat bodies of bacteria a deep blue-black color, and this dye is recommended as superior to the other Sudans.

The method used in staining the bacteria was to suspend a loopful of the cells in a drop of the stain solution and to prepare flat wet mounts. The organisms giving positive fat tests with Sudan black B included *Bacillus cereus*, *Bacillus mycoides*, *Azotobacter beijerinckii*, *Rhizobium leguminosarum*, *Mycobacterium avium*, *Mycobacterium leprae*, *Oospora lactis*, *Bacillus tumescens*, water spirilla, and fungi.

INTRODUCTION

The use of the Sudans as fat stains dates back to 1896, when L. Daddi introduced Sudan III as a specific stain for fat. L. Michaelis, in 1901, reported on his synthesis of Sudan IV, which had similar staining properties and was related to Sudan III. French (1926) reported oil red O, (*Syn.*, Sudan II) as the most brilliant of the Sudan fat stains.

The above mentioned Sudans have long been recognized as the stains of choice when testing cells for fat. Recently, however, Sudan black B has been proposed as having staining properties equal to the other Sudans. It was introduced by Lison (1934) as a specific fat stain for the detection of lipids in tissue sections, and is a dyestuff of the phenyl-azo-naphthyl-azo-naphthyl type. Recently, Leach (1938) proposed 50% diacetin and distilled water as an improved solvent for Sudan black B, and also presented a method of using it to stain lipids in sections of intestinal epithelium.

Since the stains of the Sudan series readily stain cell fats, an investigation was conducted to determine the ability of Sudan black B to stain the fats of bacteria and to find a more suitable solvent for the dye, other than those already proposed, which would more readily adapt itself for the staining of lipids in bacteria.

The method of staining the fats of bacteria is to suspend the organisms in the dye and prepare flat wet mounts. For this procedure

it is of value to have a stain which will readily suspend the bacteria in question and not cause precipitates or plasmolysis of the cells due to evaporation of the dye solvent. The solvents found to meet these requirements are 70% alcohol, 50% acetone and water, 50% dioxan and water, and ethylene glycol.

MATERIALS AND METHODS

The Sudan black B used during this study was furnished by the National Aniline and Chemical Company, New York City. The staining solution was prepared by dissolving 0.25 g. of the dry Sudan black B powder in 100 ml. of 70% alcohol at room temperature. Solution of the dye takes place almost immediately. The staining solution as described by Leach was prepared by adding excess of the dye to equal amounts of diacetin and distilled water followed by two days incubation at 55° C. and filtering before using.

Saturated solutions of Sudan black B in 50% acetone and distilled water, 50% dioxan and distilled water, and ethylene glycol¹ were prepared. For best results it is advisable to let the solutions stand for several days since the acetone and dioxan require considerable time for solution to take place.

The method used in staining the bacteria was to suspend a loopful of the cells in a drop of the stain solution and prepare flat wet mounts.

The organisms tested for fat during this investigation included *Bacillus cereus*, *Bacillus mycoides*, *Bacillus tumescens*, *Azotobacter beijerinckii*, *Rhizobium leguminosarum*, *Mycobacterium avium*, *Mycobacterium leprae* (strain isolated by J. R. Kriz), *Oospora lactis*, water spirilla, and fungi. (These organisms were obtained from the stock cultures of the University of Texas at Austin:)

The *Rhizobium* and *Azotobacter* cultures were grown on yeast extract mannitol agar and nitrogen-free mannitol agar respectively. The fungi studied were isolated from the air by exposing glucose agar plates for several minutes. The other test organisms were grown on 6% glycerin infusion agar and carbohydrate infusion agar.

The cultures were studied at various periods during their growth and the presence of fat bodies within them was detected by the use of Sudan black B. All positive fat tests were confirmed by the fat stains of Eisenberg (1909), by staining with Sudan II, III, and IV, by negative fat stains, and by unstained vital preparations.

¹The ethylene glycol used as a dye solvent should have a boiling point of 195-7° C., since if less pure it will not completely dissolve Sudan black B.



PLATE I.

Fig. 1. *Bacillus cereus* from a 48-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

Fig. 2. *Bacillus tumescens* from a 32-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.



PLATE II.

Fig. 3. *Oospora lactis* from a 48-hour culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in 70% alcohol.

Fig. 4. Water spirillum from a 12-day culture on 6% glycerin agar, showing fat bodies stained with Sudan black B dissolved in ethylene glycol.

OBSERVATIONS AND CONCLUSIONS

All the bacteria used in this study gave positive results when tested for fat by the use of Sudan black B. Fat bodies could be detected in all the organisms within 24-48 hours; however, the *Mycobacteria* gave better results if tested for fat after 7-14 days incubation. The fat droplets appear as blue-black bodies in a clear colorless cytoplasm. (Figs. 1, 2, 3, and 4). At times the organisms may assume a beady or chain-like appearance, since only the stained fat bodies are visible (Figs. 1, 2, and 4) while the cell membrane is indistinct or not visible at all.

The fat bodies stain a deep blue-black color when stained with the 70% alcohol staining solution; they assume a blue color when ethylene glycol, 50% dioxan and distilled water, and 50% diacetin and distilled water are used as the dye solvents. When Sudan black B is added to 50% acetone and distilled water, a red solution is formed which stains the fat bodies of the bacteria red; this is due to the fact that many organic dyestuffs have an entirely different color in organic solvents and only give their true color when the solvent is evaporated.

The most brilliant fat tests were observed when the bacteria were stained with the dye solutions using either 70% alcohol or ethylene glycol as the solvent. Ethylene glycol seems to be especially suitable as a dye solvent for Sudan black B, since it does not tend to evaporate when making vital mounts, and in addition has the property of taking on moisture, thus permitting preparations to remain in excellent condition for several days. The other solvents gave less striking results, altho the fat bodies were stained a bright blue color. The stain solution as recommended by Leach did not give very satisfactory results, as the fat bodies stained a faint blue and at times did not stain at all. Forty per cent alcohol, which has been recommended as a solvent for Sudan black B, proved to be unsatisfactory because insufficient dye was dissolved.

Sudan black B in 70% alcohol has retained its staining ability for over six months and does not seem to have deteriorated in any way. Dye solutions using ethylene glycol, 50% dioxan and distilled water, and 50% acetone and distilled water as solvents, also have kept their staining abilities over long periods of time. Frequently a dark precipitate is observed in the wet-mount preparations; however, since it is usually in the background and beyond the focal level of the cells, it is of no objectionable consequence. Therefore, it can be concluded that the dye solvents tried in this study do not tend to deteriorate upon aging or to cause undesirable precipitates or to give poor staining reactions.

From the observations made during this study, Sudan black B is to be preferred to Sudan II, III or IV as a bacterial fat stain. The solubility of the Sudan black B in the various solvents tried, as well as the slight solubility of the other Sudans in similar solvents, is a matter of practical importance. The microscopical picture obtained when using Sudan black B as compared to the other Sudans would indicate that Sudan black B presents a less confusing picture and is equally specific for fat substances.

SUMMARY

Sudan black B dissolved in 70% alcohol or ethylene glycol stains the fat bodies of bacteria a deep blue-black color, and is recommended as superior to the other Sudans.

The author wishes to express his appreciation to Dr. I. M. Lewis, Department of Botany and Bacteriology, University of Texas, for his helpful advice and criticism during this work.

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A SIMPLE TECHNIC FOR IN TOTO STAINING OF TARSAL AND SEBACEOUS GLANDS

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In toto or bulk staining with Sudan IV as used by Herxheimer is an efficacious method for delineating tarsal and sebaceous glands. Altho Sudan III and Sudan IV have long been used for staining fatty substances in general, the lack of references to the application of these stains for purposes referred to above indicates that the demonstration of entire tarsal and sebaceous glands is not in general use. Fatty substances in general stain more deeply with Herxheimer's stain than with Sudan III.

The eyelids and skin removed from animals (dog, ox, cat and human cadavers) were fixed in 10% formalin for 24 hours or longer and then run thru 50% alcohol (ethyl) into 70% alcohol. Care should be taken to wash out the fixing fluid thoroly with several changes of 70% alcohol. This will avoid the formation of a precipitate after the tissue is placed in the stain.

Cadavers, when received at the Indiana University anatomical laboratories, have already been injected with a preservative, the ingredients of which are unknown. The eyelids and skin removed from them for purposes designated above are fixed in 10% formalin before they are finally injected with a mixture of equal parts of alcohol, glycerin and phenol.

To delineate the tarsal glands clearly, the tissues covering the tarsus should be removed. This can be easily done by submerging the eyelid in 70% alcohol in a large Petri dish, the bottom of which has been coated with a layer of paraffin about 5 mm. thick, and by pinning the lateral and medial margins of the eyelids to the paraffin. Care should be taken not to insert the pins thru the tarsus. When the eyelid is thus anchored, the skin, orbicularis oculi muscle and the palpebral conjunctiva can be removed easily from the tarsus with a small pair of curved scissors and a pair of fine forceps. The use of binocular magnifying lenses with a long working distance is an aid in removing the excess tissue. A binocular loupe is very satisfactory to use with this type of work.

If it is also desirable to display the large sebaceous glands that open into the hair follicles of the eyelashes, the epidermal layer of the skin near the margin of the eyelids should be removed.

For the delineation of the sebaceous glands, free-hand vertical sections 1 to 2 mm. thick are made from any region of the integument where these glands are present (scalp, skin of general body surface). The sections are more easily made from fixed than from fresh material. Whole mounts of skin 12 mm. square or larger also make interesting preparations.

The formula for Herxheimer's stain¹ is as follows:

70 parts of absolute alcohol (ethyl),
20 parts of a 10% solution of sodium hydroxide,
10 parts of distilled water,
Sudan IV to saturation.²

The procedure for staining is as follows:

1. Transfer tissue from 70% alcohol to Herxheimer's stain. Staining is accomplished in 12-24 hours or in less time if the pieces of tissue are thin.
2. Wash out excess stain with repeated changes of 70% alcohol until the glands are sharply delineated.
3. Transfer to glycerin. The tissues surrounding the glands become semitransparent. The preparation may be kept permanently in glycerin, or may be mounted in glycerin jelly. Of the several formulae for glycerin jelly, Brandt's formula³ was used: melted gelatin 1 part; glycerin 1½ parts; and a few drops of carbolic acid.

Tarsal and sebaceous glands stained by this method seven years ago have not faded and make beautiful whole mount preparations to supplement the study of these glands in ordinary microscopic preparations.

¹Enzyklopädie der Mikroskopischen Technik, Dritte Auflage, Bd. 1, 729-30. Urban und Schwarzenberg, Berlin-Wien.

²The dye used for staining the majority of the specimens was labeled "Scharlach R" and purchased a good many years ago from the Harmer Laboratories of Lansdowne, Pa. For some of the recent specimens a lot marked "Scarlet R" obtained from the Coleman and Bell Co. was employed. It was subsequently pointed out by the Chairman of the Biological Stain Commission that altho the name "Scarlet R" denotes an entirely different type of dye to the dye industry it is often incorrectly employed in the medical literature as a synonym of Sudan IV. He accordingly furnished a sample of a dye sold under the latter name by the National Aniline and Chemical Co. and certified by the Stain Commission; this sample was tried and gave equally good results.

³LEE, BOLLES., 1937. The Microtomist's Vade-mecum, 10th ed., edited by J. B. Gatenby and T. S. Painter. Churchill, London. (See p. 223).

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently
the technic in each instance is given in as much detail as possible.

J. A. de Tomasi

Abstract Editor

BOOK REVIEWS

BECKER, ELERY R. and ROUDABUSH, ROBERT L. Brief Directions in Histological Technique. 6 x 8½ in., 80 pp. Paper, with spiral binding. Collegiate Press, Inc., Ames, Iowa. 1939. \$1.00.

According to the authors' statement in the preface, "This manual was prepared especially for use by classes in histological technique in the Zoology and Entomology Department at Iowa State College." Altho the book makes no pretention of having general applicability, it may prove useful in some other laboratories where an inexpensive handbook of this sort is desired. The writers give detailed directions for one standard histological procedure (Zenker-paraffin with Delafield's hematoxylin), and follow this with alternate procedures for the various steps involved. The chapter dealing with the standard procedure is about 18 pages long; and this is followed by five shorter chapters dealing with the alternate procedures for fixation, dehydrating, imbedding, staining, and mounting, respectively. A final chapter deals with special methods for tissue or microorganisms to which the more general histological technic is not applicable.—*H. J. Conn*

MICROSCOPE AND OTHER APPARATUS

MELEZER, N., and VENKEI-WLASSICS, T. Die Quecksilberhochdrucklampe als Lichtquelle für Fluoreszenzmikroskopie und Mikrophotographie. *Zts. wiss. Mikr.*, 56, 202-10. 1939.

The author suggests the use of a new tungsten-mercury-vapor lamp developed by the Simons Company in place of the expensive low voltage ultraviolet quartz lamps or the unsatisfactory ultraviolet arc lamp using iron electrodes. These lamps, designed on the general plan of the ordinary tungsten incandescent lamp with Edison base, burn evenly emitting a constant source of light having a wave length between 310-390 m μ . They operate on 220 volts A. C.—*J. M. Thuringer*.

SCOTT, G., and PACKER, D. M. The localization of minerals in animal tissues by the electron microscope. *Science*, 89, 227-8. 1939.

Partial absorption of an electron beam is known to give rise to profile pictures of bacteria, or epidermal cells. Such a beam can also be used to cause thermoionic excitation of electrons on metallic surfaces. This principle is applied here in an attempt to localize minerals in sections of animal tissues. Sections are ashed at greatly reduced pressure on the surface of a cathode coated with Ba and Sr in the electron microscope. As most of the inorganic elements in tissues are excited to thermoionic emission at relatively specific temperatures, a differentiation and localization of at least Mg and Ca is thus made possible. A picture of cellular structures in striated muscle, gastric mucous membrane, and nerve tissue can then be obtained.—*J. A. de Tomasi*.

ZELLER, A. Ein neues Kanadabalsamfläschchen. *Zts. wiss. Mikr.*, 56, 211. 1939.

A simple and practical balsam bottle is described having a large ground glass cover. An ingenious perforated groove around the neck of the bottle, beneath the cover, permits a return flow of any balsam spilled.—*J. M. Thuringer*.

MICROTECHNIC IN GENERAL

LANDAU, E. Appareil permettant la déshydration et l'enrobage d'une pièce dans le vide, sans recourir aux substances chimiques. *Bull. d'Histol. Appl.*, 16, 18-8. 1939.

The author's apparatus for fixing fresh tissues by drying while freezing is described. With this apparatus, fresh, unfixed tissue can be dehydrated without employing any of the usual chemical reagents and with no shrinkage of the tissue. Dehydration is accomplished by a system of three pumps giving an almost complete vacuum, while a gas refrigerating system lowers the temperature to about -25°C. During this process the tissue may rest upon paraffin which can then be melted by means of a warm water bath. The tissue (still in the vacuum) is thus automatically immersed in paraffin. Infiltration and embedding are completed in 15-20 min.—*M. Noble Bates*.

MILOVIDOV, P. Die Anwendung der Azetokarmin-Methode für die Färbung von fixierten Mikrotomschnitten. *Zts. wiss. Mikr.*, 56, 67-9. 1939.

The technic recommended has been employed for material fixed in each of the following fluids: Flemming, Némec IIIa, Nawaschin, Némec-Milovidov, Némec I, sublimate-acetic, alcohol, etc. Deparaffinized sections are covered with a few drops of Schneider's aceto-carmine and gently heated for 1-2 min. (without boiling). After cooling, they are rinsed in water and transferred thru 96% alcohol, abs. alcohol, xylene, to Canada Balsam. Best cytoplasmic staining is obtained after alc. or sublimate-acetic fixation. The best general results are obtained with Flemming's, Némec's IIIa, and Nawaschin's fluid. This method has the advantages of rapidity and permanency.—*J. M. Thuringer*.

ROBINSON, B. G. A note on mounting thin celloidin-sections. *J. Roy. Micro. Soc.*, 59, 79. 1939.

Some of the difficulties encountered when mounting thin sections of material embedded in celloidin and paraffin wax can be eliminated by the following method. Infiltrate the material in the usual way. Then immerse the trimmed block in abs. alcohol until perfectly transparent; harden in xylene and transfer to the waxes. Float ribbons of sectioned material on slides previously smeared with albumen, stretch them on the hot-plate, drain and immediately store in a closed glass vessel containing cotton-wool soaked in ether and a small dish of CaCl₂. The ether vapor flattens the sections and the CaCl₂ dries them. Sections may be stored indefinitely in such a vessel. Before the ether, alcohol and staining baths, a photographic squeegee-roller may be lightly rolled over the slides; the slides are then able to withstand immersion in watery reagents for several days without the loss of a single section from the series.—*H. E. Finley*.

SUTER, HANS. Über die Eignung der Schlierenmethode zur Messung osmotischer Zustandsgrößen. *Protoplasma*, 31, (3), 421-53. 1938.

The author presents in detail the application to biological material of the Töpler-Schlieren method (Töpler, A., Ann. d. Phys., 4, 838. 1904) which depends upon the diffusion of substances of different refractive indices in a solution. A leaf of *Sempervivum tectorum* is carefully removed and the cut base of the leaf sealed with paraffin after the epidermis has been carefully stripped off. The leaf is then placed in water or in a sugar solution, and by appropriate illumination, striae or the so-called Töpler-Schlieren can be detected in accordance with diffusion gradients and density changes in the medium over the exposed cell sap.—*Robert Chambers*.

WOLF, JAN. Über die Herstellung mikroskopischen Präparate der Oberfläche verschiedener Objecte mit Hilfe der Adhäsionsmethode. *Zts. wiss. Mikr.* 56, 181-201. 1939.

The author describes the production of microscopic preparations of various surfaces with the aid of the "adhesion" method. This interesting new process utilizes celloidin casts of surfaces which may be examined by transmitted light because the images develop at the point of contact of air with the differently refracting celloidin medium. To obtain a cast of the surface epithelium of the

epidermis, the following procedure is used: (1) Paint the dry surface with a thin coat of celloidin and allow to dry from $\frac{1}{2}$ to 1 min. (2) Place a piece of transparent cellophane tape over this area, avoiding the formation of air bubbles between the two layers. (3) Remove tape and celloidin with a quick movement. (4) Place on coverglass (adhesive surface down) and mount on glass slide. It may be noted that it is the under or inner surface of the cast that becomes visible on examination. The results are very interesting and instructive. The method permits modifications, i.e., surface cells may be removed *in toto* and examined against the celloidin matrix or removed with adhesive tape alone and transferred to slides for examination in transmitted light with or without staining.

The method promises to become useful in histology, zoology, botany, pharmacognosy, and criminology.—*J. M. Thuringer.*

DYES AND THEIR BIOLOGICAL USES

COLLANDER, R., and VIRTANEN, E. Die Undurchlässigkeit pflanzlicher Protoplasten für Sulfosäurefarbstoffe. *Protoplasma*, 31 (4), 499-507. 1938.

The authors champion the findings of Plowe (*Protoplasma*, 12, 196. 1931) and of Chambers and Kerr (*J. Cell. Comp. Physiol.*, 2, 105. 1932) that sulfonic acid dyes, altho highly diffusible, do not penetrate living cells. The present authors used varying concentrations of light green, acid fuchsin, and orange G in aq. media buffered with citrate and phosphate to pH 1.9 to 8.5. The material they used was the mycelium of *Aspergillus niger* which had been claimed by Bunning (*Flora N. S.*, 31, 87. 1936) to take up these dyes. They also found that orange G and cyanol are not taken up by *Tolyphelopsis stelligera* even from concentrated solutions.—*Robert Chambers.*

HÖBER, R., and BRISCOE, P. M. Correlation between secretion of dyestuffs by the kidney and molecular structure of these dyes. *Proc. Soc. Exp. Biol. & Med.*, 41, 624-6. 1939.

Some 30 mono-azo-sulfonic acid dyes were tested by perfusing isolated frogs' hearts with Ringer's solution and then with 0.0005% dye solutions. The position of the sulfonate group appeared to be the controlling factor in secretion or failure to secrete the dye. The suggestion is offered that, if sulfonate groups are attached to only one-half of the molecules, the sulfonated half may be hydrophilic and the other half may be hydrophobic and organophilic, permitting anchoring between the cell and surroundings as the first step in penetration.—*M. S. Marshall.*

KEMPTON, R. T. Differences in the elimination of neutral red and phenol red by the frog kidney. *J. Cellular and Comp. Physiol.*, 14, 73. 1939.

The excretion of phenol red by the normal and narcotized frog kidney has been studied in relation to changes in pH of the urine. It was found that in contrast to that of neutral red, the phenol red elimination was unaffected by changes in urinary pH. As with neutral red, phenol red elimination was stopped in the narcotized kidney. This inhibition of phenol red elimination is explained by an interference with cell activity, whereas that of neutral red is apparently caused by the alkalinizing of the urine subsequent to the stopping of acid excretion.—*L. Farber.*

KRAJIAN, ARAM A. A new frozen section method for the preparation of permanent frozen sections of loose texture tissues. *Amer. J. Clin. Path., Tech. Suppl.*, 3, 189-92. 1939.

Add 2 cc. of formalin to 50 cc. of fresh uncontaminated pooled human or animal serum. This keeps several weeks at room temp. Fix loose texture tissues in 10% formalin 24 hr. or longer. Trim and wash to remove formalin. Put in serum and keep in a warm place (e.g., top of paraffin oven) over night. Cover any floating sections with absorbent cotton. Decant excess of serum and add dioxan. Stand 3-5 hr. or until completely coagulated. Freeze, cut, stain and mount as usual. Preserve blocks in 10% formalin; dioxan causes shrinkage. For emergency, put thin blocks of formalin fixed tissue in serum in a paraffin oven at 56° C. 1-2 hr. Coagulate with dioxan 1 hr.—*George H. Chapman.*

YOE, JOHN H., and BOYD, GEORGE R. Patent blue V as a pH and redox indicator. *Ind. Eng. Chem., Anal. Ed.*, 11, 492-3. 1939.

An aq. solution of patent blue V may be used as an indicator for the colorimetric determination of pH over the interval 0.8 to 8.0. The colors range from yellow thru green to blue and are stable for periods up to 5 days, after which a very slight fading may be detected. The change in color is pronounced and the pH of a solution within the range given above, can be determined to 0.1 of a pH-unit by comparison with standards made up at intervals of 0.2 pH. The same dye sample must be used for both standards and unknowns because solutions of the dye from different sources often exhibit slightly different colors at a particular pH-value.

Patent blue V can also be used as an oxidation-reduction indicator in certain volumetric methods. Altho it cannot be used with $K_2Cr_2O_7$ or in the presence of HCl, it can be used with $KMnO_4$ or $CeSO_4$ if all HCl has been removed. The oxidation-reduction potential of the purified indicator was 0.78 volt. This value corresponds almost exactly to the potential at the equivalence point in the ferrous-ferric system—namely, 0.77 volt.—A. P. Bradshaw.

ANIMAL MICROTECHNIC

BAYLEY, J. H. Differential staining methods for formalin-fixed human pituitary gland. *J. Path. & Bact.*, 49, 261-8. 1939.

The technic described for differential staining of the granule cells in the anterior lobe of the pituitary has the advantages of being applicable after ordinary formol fixation, of permitting good staining of basophil granules, and of eliminating mitochondrial staining. The following are considered the most useful and permanent methods.

General procedure: Fix whole gland in 10% neutral formol-saline for 12 hr., bisect and replace in formol for a further 24 hr. Prepare paraffin sections as usual; xylene, 2 min.; abs. alcohol, 1 min.; sat. aq. $HgCl_2$, 2 min. (to improve later staining); Lugol's iodine, 2 min. To avoid staining mitochondria, bring sections to dist. water; 0.5% $KMnO_4$, 1 min.; 5% oxalic acid, 2 min.; rinse well with tap-water. Stain 2 min. with hot but not boiling 1% aq. acid fuchsin (Grubler). Rinse in dist. water. Stain 1-3 min. in the following solution: Acid violet (Revector, supplied by Vector, Ltd.), 1 g.; orange G, 0.3 g.; dist. water, 200 cc. Rinse in dist. water. If overstained in the acid violet mixture, differentiate with 80% alcohol or in extreme cases with Lugol's iodine. Rinse; dehydrate in abs. alcohol, 2 min.; xylene, 2 min.; mount in balsam. Eosinophils are crimson, basophils dark blue, chromophobe cells grey, erythrocytes crimson, and connective tissue grey.

For connective tissue proceed as before until staining. Stain 5 min. in hot but not boiling 1% aq. acid fuchsin; rinse; stain 1-3 min. in the following: acid violet (Revector), 1 g.; orange G, 0.5 g.; acid green (Revector), 1 g.; dist. water, 200 cc. Rinse; differentiate with Lugol's iodine (5 sec.-1 min.); abs. alcohol, 2 min.; xylene, 2 min.; Canada balsam. Muscle is dark red, fibrous tissue dark blue.—S. H. Hutner.

CARTER, W. The use of prontosil as a vital dye for insects and plants. *Science*, 90, 394. 1939.

Corn seedlings, with or without roots, take up neoprontosil (a red dye) very rapidly. Their leaves show red streaks within even a few seconds, and insects, like the corn leafhopper, will show presence of the dye in their tissues after feeding on dye-saturated leaves for a day or two.—J. A. de Tomasi.

DONAGGIO, A. Dimostrazione dell' esistenza di una lesione organica reversibile nell' azione degli anestetici sulle fibre nervose centrali e periferiche. *Arch. exp. Zellforschung*, 22, 171-80. 1939.

The following method is said to leave only the anesthetized nervous tissue stained: Fix 86 hr. in Zenker's fluid; rinse in dist. water; place in amber colored aq. iodine (a few drops of tincture of iodine in dist. water) for 48 hr.; rinse in dist. water; place in 2.5% $K_2Cr_2O_7$, 4-5 min. at 37 C.; dehydrate in a series of alcohols; embed in celloidin; section at 20μ ; immerse sections in Lugol's solution (5g. KI,

0.5g. I₂, 5g. water, 45g. 90% alcohol) to eliminate traces of ppt.; wash in 95% alcohol for 1 hr.; pass thru 70% and 50% alcohol to dist. water, a few min. in each; stain in stannic hematoxylin (add 1% aq. hematoxylin at least a week old to an equal amount of 20% aq. SnCl₄) for six hr.; partially decolorize by Pal's method (0.1% KMnO₄, followed by a mixture of 1 g. oxalic acid, and 1 g. K₂SO₃, or preferably Na₂S₂O₃, in 200 cc. dist. water); examine under low power until the color is removed from the normal tissue.—V. W. Kavanagh.

GOMORI, G. The effect of certain factors on the results of silver impregnation for reticulum fibers. *Amer. J. Path.*, 15, 493-5. 1939.

The impregnation of reticulum fibers by ammoniated alkaline silver reagent (Gomori, *Amer. J. Path.*, 13, 993-1002. 1937) is only slightly affected by fixation. The following fixing fluids were used with organs from man, dog and guinea pig: alcohol, Carnoy's fluid, formalin-alcohol (1:5), formalin (1:10, presumably aq., but not stated), Bouin's, Orth's, Zenker's, Stieve's, and Zenker-formalin (9:1). Carnoy's gave deep black staining of reticulum fibers with gray nuclei and almost unstained cytoplasm. Cytoplasm, but not nuclei, were stained after Bouin's; other fixatives gave results similar to formalin. Exhaustive oxidation of the sections by 2 or 3 treatments with acid KMnO₄ (0.5% H₂SO₄ added to 0.5-1% KMnO₄) alternated with decolorization by a 1-3% solution of K₂S₂O₅ intensified the differentiation between reticulum fibers and other tissue elements. Length of time of fixation in formalin from 12 hr. to several months did not affect staining, neither did decalcification with either nitric or sulfosalicylic acid. Thin sections, 8μ or less, are preferred.—H. A. Davenport.

JACOBSON, W. The argentaffine cells and pernicious anemia. *J. Path. & Bact.*, 49, 1-19. 1939.

The granules of the argentaffine cells of the gastro-intestinal tract may be stained by taking advantage of their ability to reduce silver salts and to couple with diazotizing agents. For both methods tissue may be fixed in 10% formol-saline or 10% neutral formal, dehydrated with alcohol, cleared in cedarwood oil or in methyl benzoate plus 2% celloidin, and imbedded in paraffin.

The following modified Masson-Hamperl silver staining technic was used: Wash deparaffinized sections in glass-dist. water (10 min. is enough if two changes of water are used); place 12-24 hr. in Fontana's solution (prepare by adding NH₄OH to 5% AgNO₃, until the precipitate is dissolved, then add more AgNO₃ drop by drop until the fluid shows a slight persistent opalescence; glass-dist. water must be used); wash slides 1 min. in glass-dist. water; 5% Na₂S₂O₃, 1 min.; tapwater, 10 min. Only the granules of the argentaffine cells appear black. Nuclei may be counterstained with carmalum and the sections permanently mounted in Canada balsam.

An alternative quicker diazo method is as follows: prepare diazotizing solution by dissolving small amount of *p*-nitro-methoxybenzene diazotize (Kernechtrotsalz B) in dist. water to produce a light yellow solution. Alkalinize with a small amount Li₂CO₃. About 1/2 min. later pH 10-11 is reached, the color changing to dark orange-yellow. Take sections from dist. water; immerse in stain, 30-40 sec.; wash 1 min. in dist. water. Granules of argentaffine cells are dark red against a yellow background. Nuclei can be counterstained with hemalum.—S. H. Hutner.

KATÔ, HIDEHARU. Über den Einfluss der Fixierung auf das Hirngewicht. *Folia Anat. Jap.* 17, 237-97. 1939.

This study on the influence of fixation on brain weight was based on the examination of an extensive series of mouse, rat, cat, dog, monkey, and human brains fixed respectively, in Muller's, Helly's, Orth's, Zenker's, Ciaccio's, Carnoy's fluids, in alcohol and in 10% or 20% formalin.

It was found that Müller's, Helly's, Zenker's fluids and formalin produced an increase in weight while Carnoy and watery alc. solutions caused the brains to lose weight. Ciaccio's fluid did not influence the weight appreciably one way or another. Formalin fixation of human brains produced an average increase in weight of 10%-12% in 8-4 days, whereupon the weight remained constant for about 3 months, then diminished slowly to an average of 1.9% above their original weight after 24 months fixation.

The author stressed that brains of various animals reacted in a definite individual manner. The results are given in 37 tables and 7 graphs.—*J. M. Thuringer.*

LEPLAT, G. Des avantages de la glycérine dans la déshydratation des tissus conjonctifs et des os, avant l'enchaînement. *Bull. d'Histol. Appl.*, 16, 118-21. 1939.

Impregnation with glycerin is recommended to counteract the hardening and shrinking effect of the higher alcohols upon tissues which previously have been decalcified.

Good sections of the acoustic epithelium with Reissner's membrane always intact are obtained when (after decalcification with an aq. solution of HNO_3 , treatment with Na_2SO_4 , and washing) the labyrinth is placed for 2-3 days in each of the following: (1) a solution of 1 part glycerin to 3 parts dist. water, (2) a solution of equal parts glycerin and water, (3) pure glycerin. Transfer for 2 hr. to abs. alc. to complete dehydration and to remove the glycerin. Place in an abs.-alc.-ether mixture. Embed in celloidin.

Difficulty in obtaining serial sections of bone dehydrated in the usual manner is overcome if glycerin is employed in the process. Pieces of bone decalcified, washed, and run up to 50% alcohol are treated as follows: (1) 50% alcohol, 6-10 hr. (2) Anhyd. glycerin and 70% alcohol in equal parts, 24 hr. (3) Pure glycerin, two changes in 24 hr. (4) Abs. alc., 2 hr. (5) Clear in cedar oil and embed in paraffin.

In other tissues which are difficult to section serially (but which do not contain bone), the author finds that a certain quantity of glycerin added to the higher alcohols (70%-95%) in the dehydration process results in easier and more regular sectioning.—*M. Noble Bates.*

PERDRAU, J. R. Ammonium molybdate as a mordant for Mann's stain and the Weigert-Pal method. *J. Path. & Bact.*, 48, 609-10. 1939.

For consistent Mann staining, treat material fixed in mercuric mixtures as follows. Place sections, Hg-free, in 2% ammonium molybdate overnight at 37° C. Wash thoroly in dist. water for 5-10 min.; leave in Mann's mixture overnight. Wash in dist. water and differentiate in 70% alcohol tinged with a trace of orange G, controlling the process under the low power of the microscope. Dehydrate, clear and mount. Overmordanting and overstaining is impossible.

With Mann's original staining solution the following results are obtained: Chromatin, nuclear membrane, cell membrane and connective tissue are blue; nucleoli, erythrocytes, cytoplasmic inclusions and many cell granules bright red; the rest pale pink. With other fixatives the background may be blue instead of pink.

Old bichromate-hardened frozen sections of nervous tissue that have been kept a long time in alcohol after hardening can be made to stain properly, myelin included, by mordanting in ammonium molybdate.—*S. H. Hutner.*

POURSINES, Y. Techniques de coloration myélinique et cellulaire (type Nissl) du tissu nerveux, sur coupes à la paraffine provenant d'un même bloc. *Bull. d'Histol. Appl.*, 16, 128-34. 1939.

The steps of a myelin technic for paraffin sections are described. A 4-6 day ferric impregnation of the tissue by a 5% aq. solution of ammoniacal iron alum is done before embedding. (To secure a good impregnation, the formalin-fixed tissue has to be treated first with 95% alcohol followed by an ether-alcohol mixture).

Mounted sections are mordanted in 5% iron alum solution, stained with Regaud's hematoxylin, and differentiated with KMnO_4 and 22% iron alum. Myelin sheaths are stained dark gray or black.

Other or alternate sections cut from the same block may be stained to show Nissl bodies, or may be stained with hematoxylin and eosin, by first treating them with oxygenated water or with HCl , so as to modify the iron compounds deposited in the tissue during the ferric impregnation. Directions are given for this treatment and the staining with Unna's polychrome blue.—*M. Noble Bates.*

SCHROEDER, KURT. Eine weitere Verbesserung meiner Markscheidenfärbemethode am Gefrierschnitt. *Zts. gesam. Neurol. u. Psychiat.*, 166, 588-93. 1939.

For staining myelin sheaths in frozen sections, the following technic is recommended:

Fix nervous tissue in formalin as usual. Cut 20-30 μ frozen sections. Mordant 24 hr. at 37° C. in a mixture 1 vol. of Muller's fluid and 2 vol. of Weigert's rapid mordant. (Composition of the rapid mordant is not given, but presumably consists of CrF₃ 2.5 g., water 100 cc., heated to boiling in a covered vessel, with 5 cc. glacial acetic acid and 5 g. of copper acetate added in the order given). Wash quickly in dist. water and transfer to the staining solution made as follows. Add 3 cc. of any kind of 10% alc. hematoxylin solution to 100 cc. dist. water and boil 5 min. Cool and add 3 cc. sat. aq. soln. LiCO₃. Make up to 100 cc. if there is much loss in boiling. Stain sections for 12 hr. or longer at 37° C. Wash well and treat with 0.25% aq. KMnO₄ about 1/2 min. Wash twice in dist. water during the next minute and differentiate in a solution made by mixing equal parts of 1% aq. oxalic acid and 1% aq. K₂SO₃. Keep the sections in motion during differentiation and change the solution after 1/4 to 1/2 min. Continue the differentiation until the gray matter is clear, then soak 15 min. in sat. aq. LiCO₃, 1 cc., plus tap water, 100 cc. Wash thoroly, dehydrate and mount in balsam. The method can be used for gelatin embedded frozen sections with the following modifications: Mordant 5 days at 37° in Muller's fluid, stain several days, use 1% KMnO₄, and double the concentration of the differentiator.—H. A. Davenport.

SPEK, J. Studien über die Polarität der Larven der Kalkschwämme. *Protoplasma*, 30 (3), 352-72. 1938.

The author used traces of dil. solutions in sea water of brilliant vital red (Grubler), Nile blue sulfate B, and brilliant cresyl violet on the blastulae of *Sycandra setosa* and *Leucandra aspersa*. Particularly with brilliant cresyl violet, he obtained striking color differences (from blue to red) in the different cells of the blastulae. These color differences he ascribed to differences in pH of the cytoplasm. His illustrations indicate that the color appears in the granular contents of the cells.—Robert Chambers.

SPOERRI, ROSETTE. A new material for mounting nerve tissue sections in paraffin for silver staining or restaining. *Science*, 90, 260. 1939.

Egg albumin is not always a satisfactory fixative for nerve tissue sections. The following starch paste was found reliable: suspend 1 g. starch in 10 cc. cold water, add 20 cc. boiling water, and stir until homogeneous. Add 2 drops HCl, and boil 2-5 min. After cooling, preserve with a small crystal of thymol. Use like albumin, allowing the sections to dry on the slide 3 days at 45° C.

For Ag impregnations of sections on slides, the technic is as follows: fix material in formalin, section in paraffin, and affix to slides. Dry; pass thru xylene into water, and up thru graded alcohols to pyridine for 15 min. Put in fresh pyridine overnight. Wash 10 min. in dist. water, and impregnate 3 hr. in the dark in 5% AgNO₃. Transfer for 30 min. to ammoniacal AgNO₃ (5% AgNO₃, 200 cc.; 10% NaOH, 5 cc.; with NH₄OH added drop by drop until precipitate dissolves). Wash rapidly in dist. water, soak 5 min. in 10% formalin. Wash; tone in gold, or fix 2 min. in "hypo"; dehydrate; clear; and mount.—J. A. de Tomasi.

TERRY, R. J. A thoracic window for observation of the lung in a living animal. *Science*, 90, 43-4. 1939.

An instrument has been devised suitable for observation of the superficial air sacs and alveoli in the cat's lung. It consists of a bronze cylinder with a small coverglass window mounted at one end, and provided at the other with a quadrilateral plate or flange. The instrument is placed in position thru an opening in one of the intercostal spaces in the thoracic wall, so that the end-plate rests against the inner surface of the wall while the window faces the lung. When the air in the pleural cavity is taken out by exhaustion, the lung surface comes to rest against the window and can be studied most conveniently. Observations are carried out with a binocular and a skin microscope. A motion picture of local phenomena has been obtained.—J. A. de Tomasi.

WALLART, J. Essais de coloration de l'hypophyse. *Bull. d'Histol. Appl.*, 16, 149-52. 1939.

In an attempt to distinguish more clearly between the different cells of the hypophysis, the author developed a modification of existing staining methods. After preparing the sections in the usual way, they are stained as follows: Cover with Masson's ponceau-fuchsin or 1% acid fuchsin, 5 min.; wash briefly in dist. water; add 5% phosphotungstic acid, 5-8 min.; drain the surplus without washing; add Krall's anilin blue or Hollborn's standardized anilin blue, 3-5 min. with slight agitation; rinse quickly in running water; immerse in 1% acetic acid in dist. water, 30 min.; 1% acetic acid in abs. alcohol, ½-1 min.; abs. alcohol; toluene; Canada balsam.

The results are: Chromatin of the nuclei ordinarily blue, sometimes reddish or even bright red, especially in degenerating nuclei; the nucleolus most often bright red but sometimes bluish; karyoplasm sometimes colorless, sometimes bluish to deep blue; the acidophiles red; the basophiles blue; granules of the principal cells gray-blue; erythrocytes red to blue-gray.—*Jean E. Conn.*

PLANT MICROTECHNIC

BALDWIN, J. T. Chromosomes from leaves. *Science*, 90, 240. 1939.

The following smear method for chromosomes from leaves is a modification of Warmke's technic (*Stain Techn. 10*, 101-8, 1935): Fix young leaves in Carnoy's for 5 min. or longer; pass briefly thru 95% alcohol and conc. HCl, 1:1; carry back into Carnoy's, and after a few min. smear out in the aceto-carmine.—*J. A. de Tomasi.*

MICROORGANISMS

BROADHURST, JEAN and PALEY, CHARLES. A single-dip stain for the direct examination of milk. *J. Amer. Vet. Med. Assoc.*, 94, 525-6. 1939.

This staining procedure is essentially that employed in the Newman technic for the direct microscopic count of bacteria in milk. It differs in the inclusion of basic fuchsin for the staining of the background material. This, in the opinion of the authors, makes for more accurate counts with less eye strain.

Preparation of Stain. Add 0.4 cc. of concentrated H_2SO_4 (measured accurately) to 54 cc. of 95% alcohol. Mix with 40 cc. of technical tetrachlorethane in a flask and heat to about 55° C. (no higher). Add the combined solution while hot to from 1.0 to 1.2 g. of methylene blue; shake until dye is dissolved. Add 8.0 cc. of a 1% solution of basic fuchsin in 95% alcohol. Mix well, cool, filter, and store in tightly stoppered bottles.

Directions for Use. Prepare milk smear as usual, by spreading 0.01 cc. of milk on a slide over an area of 1 or 2 sq. cm., as preferred. Dry the smear on a flat surface in a warm place within 5 min. When dry, dip the slide in the stain or flood the slide with the stain for about 15 sec. Drain off excess stain and dry while flat in a warm place. Wash in cold water until all the blue is washed out of the smear and it assumes a faint pink color. Dry and examine under an oil-immersion objective.—*A. Zeissig.*

DELAPORTE, B. Sur les acides nucléiques des levures et leur localisation. *Rev. Gen. de Botanique*, 51, 449-82. 1939.

Zymonucleic acid was extracted in relatively large quantities from living yeast cells, without affecting the nuclei at all, by washing them 2 hr. with pure water or 0.1-0.2% $NaHCO_3$ solution. With this washing, however, metachromatins granules (volutin) disappeared from the cytoplasm of *Saccharomyces ellipsoideus* and were much reduced in other yeasts. When the cells were killed with acetone, all the zymonucleic acid was extracted from the cells with the water or acetone, but still the nuclei were intact and gave positive Feulgen reactions. Thymonucleic acid extraction technics removed the material giving the Feulgen reaction from the nuclei and concentrated it in the solutions, analysis of which showed that the nuclear nucleic acid of yeasts was either thymonucleic acid or an unidentified acid very closely related to it, derived from a glucoside containing thymine, and reduced in the 2-position. The nucleus was left intact even after the second extraction and the nucleolus still stained with ferric hematoxylin.

A solution of methylene blue or cresyl blue (concentration not given), added for a few seconds either to living yeast or to smears that had been fixed in alcohol for less than $\frac{1}{2}$ hr. or by flaming, stained the small metachromatin granules in the vacuoles red or reddish violet in contrast to a pale blue cytoplasm. To complete the test Meyer's reaction was used by treating the slide stained with methylene blue with 1% H_2SO_4 for a few seconds; this removes the color except from the metachromatin granules which appear black.—*V. W. Kavanagh.*

GELEI, GÄBER VON. Neue Silbermethoden im Dienste der Protistenforschung. (Komplexsilberverbindungen). *Zts. wiss. Mikr.*, 56, 148-80. 1939.

The author has modified the methods of Achucarro by introducing other fixatives, reducing agents, and complex organic silver combinations. He has also substituted a sublimate-potassium-dichromate-alum mixture ($HgCl_2$, 7%; $K_2Cr_2O_7$, 2.5%; $AlK(SO_4)_2 \cdot 12H_2O$, 1%) for the usual formalin-sublimate mixture and developed a special tannin-silver technic which gives certain results in the impregnation of cilia and the basal apparatus.

A) Tannin-silver method: (Modification consists in using tannin as a mordant.) (1) Sensitize the living material in $FeCl_2$ solution prepared by adding 1 drop of 0.1% $FeCl_2$ solution to each ml. of culture fluid. The addition of 2 drops of 1% OsO_4 to every ml. of fixed solution improves the stain. (2) Fix in sublimate-dichromate-alum, 1 min. (3) Wash in dist. water, 2 changes. (4) Cold sat. soln. tannin (Merck) heated to 50° C., 2 min. (5) Wash in dist. water. (6) Place in 1% ammoniacal $AgNO_3$, $\frac{1}{2}$ min. (7) Rinse in several changes of dist. water. (8) Dehydrate in alcohol series. (9) Pass thru xylene to balsam. The method gives good results with various elements of the ciliates but not with the conduction apparatus.

B) By replacing tannin in the above technic with pyrogallol-carbonic-acid (2-3-4 trihydroxy benzoic acid), three new morphological elements were stained; namely, the elastic infra-ciliary reticulum, longitudinal fibrils of the excretory apparatus, and the sheath of the neuromeme.

C) Further modifications of the ammoniacal $AgNO_3$ basic method: (1) Essential requirement: a very active *Paramecium* culture at least one week old prepared either with a boiled straw-hay infusion or fresh horse manure (1 piece to 3 liters tap water). (2) Fix in sublimate-dichromate-alum solution, 1 min. (3) Wash in 2 changes of dist. water. (4) Conc. aq. soln. pyrogallol-carbonic-acid, 2-15 min. (cold sat. soln. plus a very slight excess crystals). (5) Wash in dist. water. (6) Ammoniacal $AgNO_3$ (Rio-Hortega), 1 min. (7) Wash in 2 changes dist. water. (8) Pass thru 40% alcohol, and glycerin-alcohol; mount in glycerin alcohol. The excretory organs were demonstrated with specially good results by the introduction of other organic silver preparations such as ammoniacal silver acetate, citrate, oxalate, lactate, benzoate, salicylate, and carbonate in place of Bielschowsky's ammoniacal $AgNO_3$.—*J. M. Thuringer.*

NIETHAMMER, ANNELIESE. Mikroskopische Bodenpilze als Begleiter in Früchten und Samen. *Arch. Mikrob.*, 10, 13-25. 1939.

In the course of an investigation as to the presence of fungi in seeds and small fruit, the author has developed the following technic:

Wash the fruit or seed thoroly with sterile dist. water. Section quickly, transfer the tissue pieces to a sterile slide and cover quickly with a flamed cover glass. Introduce sterile solution of Oxaminblau (further identification and strength not given) at the edge of the cover glass and allow to stain. Transfer tissue to a suitable sterile nutrient medium and allow to remain several hours. The stain carried over is not detrimental and a distinct growth of the mycelium takes place. This stain gives a good differentiation of tissue.—*Merritt N. Pope.*

STONE, W. S. and REYNOLDS, F. H. K. A practical method of obtaining bacteria-free cultures of *Trichomonas hominis*. *Science*, 90, 91-2. 1939.

The isolation of the protozoon *Trichomonas hominis* is carried out in a 16 in. capillary tube drawn from an 8 in. length of 6 mm. pyrex tubing, plugged with cotton and sterilized by dry heat. The capillary is bent into a series of small traps, in the manner of a gas analysis tube. The method follows: by means of suction, fill the tube with a sterile culture liquid (Ringer's, 1 vol.; horse serum, 8 vol.); flame and seal the distal end of the capillary, and after a 48 hr. sterility

test, inoculate the medium. Incubate at 37° C., keeping the tube vertical. The protozoa will slowly migrate toward the bottom of the tube where they can be observed under the microscope, free from bacteria. Cut off and seal the capillary in a flame, drop for 1 hr. in tincture of iodine, drain off excess, and cut capillary segments into selected culture media.—*J. A. de Tomasi.*

WALTON, SETH T. A quick and reliable method for staining Gonococcus smears. *J. Lab. & Clin. Med.*, 24, 1808-9. 1939.

The author gives a modification of the Pappenheim-Saathof stain. The modified solution is prepared as follows: Methyl green (dye content 60%), 1.0 g.; pyronine B certified, 0.2 g.; abs. methyl alcohol, 10 cc.; 2% aq. phenol, 100 cc.; glycerine C.P., 20 cc. Dissolve dyes by intermittent shaking for 2 days, employing a mechanical shaker. Fix films in the usual way, add dye to warm slides and let stand for 20-50 sec. The dye is selective for the *Neisseria* group.—*Sara A. Scudder.*

HISTOCHEMISTRY

ARENS, K. Lokaler Nachweiss von Kalzium in den Membranen des Elodeablattes mittels Natriumoleat. *Protoplasma*, 31, (4), 508-17. 1938.

Elodea leaves remain alive for 24 hours in 0.5% solution of sodium oleate. The precipitate which gradually forms on the surfaces of the cell walls is claimed to be calcium oleate since the chemical reaction of the precipitate corresponds to that of calcium oleate.—*Robert Chambers.*

MALLORY, FRANK B. and PARKER, FREDERICK, JR. Fixing and staining methods for lead and copper in tissues. *Amer. J. Path.*, 15, 517-22. 1939.

To demonstrate the presence of lead, tissues must be fixed in 95% or abs. alcohol. Formalin is worthless. The procedure recommended is: Stain celloidin sections at 54° C. for 2-3 hr., rarely longer, in the following solution: 5 to 10 mg. (not more) of hematoxylin dissolved in a few drops of abs. or 95% alcohol and mixed with 10 cc. of a freshly filtered 2% aq. solution of K_2HPO_4 . After staining, wash in several changes of tap water for 10 min. to 1 hr.; dehydrate in 95% alcohol; clear in terpineol; and mount in terpineol balsam. Lead is stained a light to dark grayish blue, and nuclei a deep blue. Another method consists of staining 10-20 min. in a 0.1% solution of methylene blue in 20% alcohol. (Sources of dyes are not stated.) Differentiate 10-20 min. in 95% alcohol. The methylene blue stain works on Zenker-fixed paraffin sections and is particularly effective when phloxine is followed by methylene blue. Copper can be demonstrated after either formalin (neutral aq.) or alcohol fixation. The same hematoxylin stain is used as for lead. Hemosiderin (Fe-pigment) stains black while copper hemofuscin stains blue. The methylene blue stain colors the Cu-pigment pale blue but does not color the Fe-pigment. The probable specificity of the staining reactions was checked on tissues of animals acutely poisoned by Pb and Cu salts.—*H. A. Davenport.*

MILOVIDOV, P. Bibliographie der Nucleal- und Plasmareaktion. *Protoplasma*, 31 (2), 246-66. 1938.

Almost twenty pages of references are listed on the subject of Feulgen's specific staining reaction of chromatin. Among these are included those dealing with the aldehyde reaction which may also occur in the cytoplasm.—*Robert Chambers.*

TONUTTI, E. Ergebnisse histochemischer Vitamin C-Untersuchungen. *Protoplasma*, 31 (1), 151-8. 1938.

The author reviews the technic of Giroud and Leblond (*Arch. Sciences et Ind.*, 435, 1936, Paris) for cytological demonstration of vitamin C as follows: Short washing of specimen in isotonic levulose solution (5.4%); transfer to 10% $AgNO_3$, containing two drops glacial acetic per cc. (up to $\frac{1}{2}$ hr.); rinse in dist. water for $\frac{1}{4}$ - $\frac{1}{2}$ hr., changing the water; transfer to 3% $Na_2S_2O_3$, $\frac{1}{4}$ - $\frac{1}{2}$ hr.; rinse in dist. water, $\frac{1}{4}$ - $\frac{1}{2}$ hr.; transfer to 70% alcohol for paraffin imbedding. These steps should be performed in a dark-room with red light. Counterstain with "Kernechtrot" and light green. Sections should be thoroly dehydrated and protected from light.—*Robert Chambers.*

STAIN TECHNOLOGY

VOLUME 15

APRIL, 1940

NUMBER 2

THE PRESENT SITUATION CONCERNING GIEMSA STAIN

Giemsa stain is well known to be a mixture of eosinates of the oxidation products of methylene blue. The basis of this stain is the product developed by Giemsa under the name of azure I. The method of manufacture of this product was turned over by Dr. Giemsa to the firm Hollborn & Sons which is the successor of one of the laboratories founded by Dr. Grübler. The exact method of preparation has never been divulged, but the product is known to be an oxidation product of methylene blue, presumably a mixture of two or more chemical compounds. MacNeal¹ indicated this product to be a mixture of two compounds which are now designated azure A and azure B. Of these, MacNeal regarded azure B as having little if any staining value.

From azure I, Giemsa prepared another product, which he called azure II, by adding to it equal parts of untreated methylene blue. An eosinate prepared from this he denoted "azure-II-eosin" and this eosinate is at least the main constituent of the dry Giemsa stain. The liquid Giemsa stain is a solution of this, with additional azure II, prepared by a formula published by Giemsa.

Following MacNeal's suggestion that azure B is of no staining value, recent American efforts to obtain an equivalent of the Giemsa products have called for the use of azure A prepared by the method of Holmes and French² and presumably free from azure B. A few American stain companies have for several years been producing Giemsa stain, with azure A its main constituent, and have been submitting the products thus prepared to the Stain Commission for certification. Most of these products have been approved because they have given excellent results when tested by the technic that has been employed by the Commission. In granting this approval, however, chief stress has been laid upon performance as a stain for fixed thin films of blood.

¹MacNeal, W. J. 1925. Methylene violet and methylene azure A and B. *J. Inf. Dis.* **36**, 588-46.

²Holmes, W. C. and French, R. W. 1926. The oxidation products of methylene blue. *Stain Techn.*, **1**, 17-26.

During the past decade, however, Giemsa stain has come into wide use for staining unfixed thick blood films for the diagnosis of malaria, particularly in surveys of the incidence of blood infection, conducted by state, county, and city health departments. Since this development has been accompanied by increasing complaints as to the behavior of American Giemsa stains, the question has arisen as to whether the stain hitherto approved by the Stain Commission is suitable for this thick film technic. Investigations of this point have been carried on with the cooperation of the U. S. Public Health Service, and indicate very clearly that only a few of the American samples are satisfactory for the technic in question.

The thick film method of malaria diagnosis was based on a technic introduced by Ross but which has been modified by others and was finally standardized in 1929 by the U. S. Public Health Service for use in malaria survey work. In this method, thick films are made by spreading 3 to 5 drops of blood over a circle of about 15 mm. diameter on a scrupulously clean slide. These films are thoroly air-dried in a horizontal position and protected from dust and insects; this ordinarily requires 18 to 24 hours at room temperature. The slides are then allowed to stand on edge for 45 minutes in a Giemsa solution diluted in neutral distilled water or with buffer solution. The latest technic recommended by the Public Health Service calls for a dilution of 1 to 50 in distilled water buffered with phosphates to a pH-level of 7.0 to 7.2. After staining, the smears are washed 5 to 10 minutes in neutral distilled water, or preferably water buffered to pH 7.0 to 7.2, in order to clear the background. By this technic the hemoglobin should be laked out of the red corpuscles and the malarial parasites should show with clear red chromatin and clear blue cytoplasm. A Giemsa stain which does not show this picture is regarded as unsatisfactory.

The laboratories of the Public Health Service report the most consistently satisfactory results with German Giemsa stain. They have used this widely for the purpose and have recommended it to other laboratories. They have tried samples of Giemsa stain prepared by various American manufacturers from time to time; but generally speaking these have not given *consistently* satisfactory results. Recently, however, on account of the impending scarcity of imported Giemsa stain, an acute demand has developed for an American product satisfactory for staining malaria parasites by the thick film technic described above.

The Biological Stain Commission has offered to help in improving this situation and has agreed to adopt the thick film technic as a

criterion of a satisfactory Giemsa stain just as soon as it is possible to give manufacturers some idea as to how to produce a stain satisfactory for this purpose. In the meantime, the coöperation of certain workers in the U. S. Public Health Service and in some of the State Health Laboratories has been made available; and all samples of this stain recently submitted to the Commission have been tested by one or more of those taking part in this coöperation.

In this way it has been found that of seven recently certified samples, three proved satisfactory by this thick film technic; namely LGe-1, GGe-5, and NGe-5. All of these were certified in 1939. GGe-5 is available in solution form, the other two in dry form. Investigations are in progress to indicate why these particular samples are superior to the others and how to standardize the manufacture of the stains so that consistently good results can always be expected. Until this is done, however, the wisest thing seems to be to urge those who use this method to purchase, when possible, one of the above-mentioned three batches of certified Giemsa stain.

Accordingly, within the last few months, the U. S. Public Health Service has sent a circular letter to state health officers in 13 of the southern states where malaria is prevalent. In this letter they call attention to the handicap arising from the present shortage of German Giemsa stain and mention the coöperation that is being undertaken with the Biological Stain Commission to determine which of the present certified products are most satisfactory. In view of the approaching malaria season (the letter continues) it seems well to get the information to the health officers at an early date. Accordingly, the certification numbers of the three satisfactory batches above mentioned are given together with the names of the manufacturers.

The Stain Commission also stands ready to furnish the names of manufacturers of these batches to anyone interested in getting the information.

This is a very empirical method of standardizing Giemsa stain. It is hoped that a more scientific method of standardization can be accomplished later. In the meantime, this statement should be of assistance to those anxious at the present time to secure a lot of stain satisfactory for the method under discussion.—H. J. CONN.



CARD MOUNTS FOR HANDLING ROOT TIPS IN THE PARAFFIN METHOD¹

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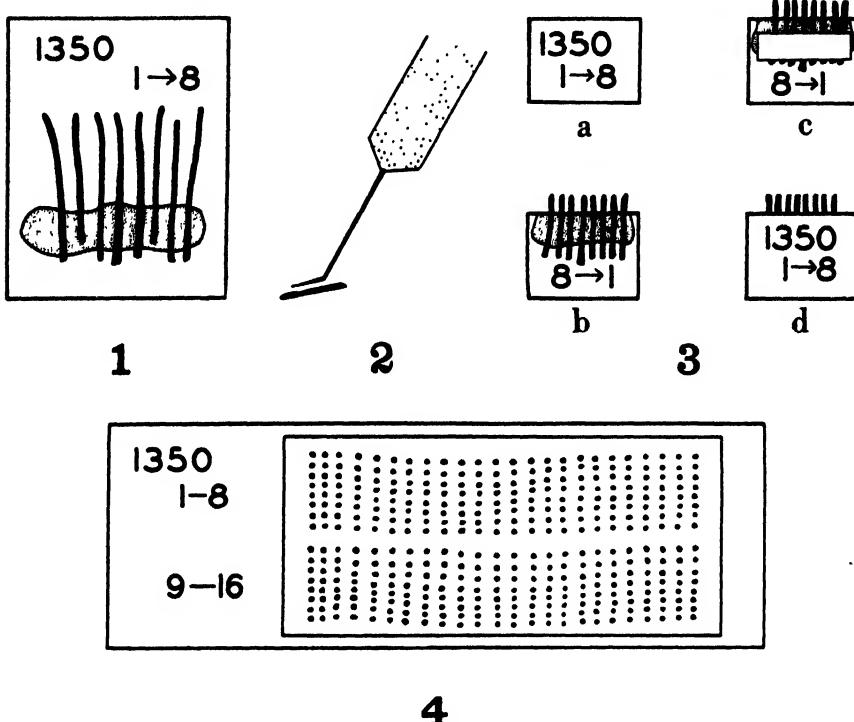
ABSTRACT.—A method is described for utilizing card mounts to facilitate the handling of root tip samples and similar material in the paraffin method. The freshly collected roots are attached to temporary card mounts having dimensions of approximately 2 cm. \times 2.5 cm. with DuPont Household Cement or a similar adhesive that hardens rapidly in the ordinary aqueous fixing fluids and is insoluble in the lower grades of alcohol. After fixation and dehydration to 75% alcohol, the roots are transferred to permanent card mounts on which they are carefully oriented for sectioning. Mucilage or glue which hardens rapidly in 85% alcohol and is insoluble in the ordinary dehydrating and infiltrating media is used in making the permanent card mounts. Detailed instructions are given for preparing and handling the card mounts, and a system of labeling the mounts is also suggested.

The necessity of handling separately numerous samples of root tips and similar material in the various steps of the paraffin method may be obviated by mounting the roots on cards which can be handled collectively in a single container. These card mounts also provide a means of orienting the roots in groups for sectioning so that a considerable number may be mounted on a single slide in an orderly arrangement without losing the identity of individual roots. Altho developed primarily for preparing root tips for chromosome counts, the technic is applicable to other types of material and may also be used to good advantage in comparing fixing, dehydrating and staining reactions.

The essential features of the method include the making of temporary card mounts (Fig. 1) at the time of fixation, followed after fixation and partial dehydration by a transfer of the roots to permanent card mounts on which they are carefully oriented for sectioning (Fig. 3). If desired the temporary mounts may be stored in-

¹Coöperative investigations of the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Department of Botany, N. Y. State College of Agriculture, Cornell University, Ithaca, N. Y.

definitely in 70% alcohol. When relatively few samples are to be taken or when many roots of the same kind are to be fixed, the temporary mounts may be dispensed with, the roots being fixed in vials in the usual manner and subsequently arranged on cards in an orderly manner for dehydrating, embedding and sectioning. For preparing



Figs. 1-4. Diagrams illustrating the preparation of card mounts and prepared slides of root tips in the paraffin method. *Fig. 1.* Temporary card mount of fresh root tips attached to the card with household cement. *Fig. 2.* Root tips detached from the temporary card mount are transferred individually to the permanent mount with a bent dissecting needle. *Fig. 3.* Steps in the preparation of permanent card mounts: *a*) One side of the card labeled to identify the individual roots; *b*) Roots with tips projecting 2-3 mm. attached to the other side of the card with glue or mucilage; *c*) A narrow strip of stiff paper is placed over the roots to secure them more firmly in position for sectioning; *d*) The finished permanent card mount as it appears from the labeled side. *Fig. 4.* Prepared microscope slide on which was mounted a ribbon from each of two card mounts containing cross sections of sixteen root tips.

the temporary mounts an adhesive is used that hardens rapidly in ordinary aqueous fixing fluids and is insoluble in the lower grades of alcohol. The permanent mounts are prepared with an adhesive that hardens rapidly in 85% alcohol and is insoluble in the higher alcohols and paraffin infiltrating media.

The following directions may be helpful in preparing and handling the card mounts.

1. Prepare temporary card mounts from small pieces of heavy paper (heavy weight filing cards are suitable stock material) with dimensions of approximately 2 cm. \times 2.5 cm. Smear the base of the card with DuPont Household Cement or some similar adhesive that hardens rapidly in the ordinary aqueous fixing fluids. Place the freshly collected roots in position on the card and add more cement, leaving at least 0.5 cm. of the tips of the roots free (Fig. 1). Invert the card with attached roots at once in the fixing fluid, keeping the cards separated for a few moments until the cement has partially hardened. This can be accomplished more readily if a shallow container is used for the fixing fluid.

2. Prepare permanent card mounts from somewhat smaller pieces of heavy paper with dimensions of approximately 12 mm. \times 15 mm. After the roots attached to the temporary mounts have been fixed and transferred to 75% alcohol, the tips of the roots are removed from the card in a petri dish containing a small amount of 75% alcohol. The card on which the roots are to be mounted permanently is first labeled on one side (Fig. 3a), and then coated on the other side with a thin layer of mucilage or glue. For this purpose the clear, amber-colored grade of Carter's or Stafford's glue, evaporated to the consistency of heavy syrup, is most satisfactory; it hardens rapidly in 85% alcohol and is soluble in the reagents ordinarily used for infiltration with paraffin wax. The root tips are then transferred rapidly, one by one, from the petri dish to blotting paper for removal of the excess alcohol, and then to the card, care being taken to orient the roots approximately as desired for sectioning transversely (Fig. 3b). Add more mucilage and place over the roots a thin strip of paper as shown (Fig. 3c). Immerse the card with attached roots at once in a petri dish containing 85% alcohol. For transferring the roots quickly from the blotting paper to the card, a bent dissecting needle applied to the moist surface of the root is very effective (Fig. 2). The final orientation of the roots on the card may be completed immediately after the transfer to 85% alcohol before the mucilage hardens. The root tips should project 2-3 mm. beyond the edge of the card and must be kept free of mucilage since it interferes with the sectioning of the roots. Very small roots have a tendency to become dried out and shriveled during the mounting process; this difficulty may be lessened by placing the card on a moist filter paper during the mounting procedure and by mounting the roots, if possible, in a cool moist atmosphere free of air currents.

3. After the mucilage has hardened, which ordinarily requires but a few minutes, the mounts are placed together in a small pyrex glass beaker and dehydrated and infiltrated in the usual manner. A revised and much abbreviated schedule² is used in this laboratory for handling the root tip mounts in the paraffin method. The mounts should be embedded with the labeled side down so that they can be identified readily in the paraffin blocks, and if they are placed in the microtome in this same position, the ribbons can be placed on the slide with the roots in an orderly sequence (Fig. 4).

With a little practice and careful adherence to the instructions given above, the average technician should have little difficulty in preparing satisfactory card mounts. The use of these mounts in our experience saves time and reduces the amount of alcohol and other reagents required for handling large numbers of roots. Their use also eliminates errors that may result from handling numerous samples individually. In addition, it is advantageous to be able to section a number of roots at one time and to mount a considerable number in an orderly manner on a single slide. This is an especially valuable feature when comparing fixing and staining reactions.

²Randolph, L. F. A new fixing fluid and a revised schedule for the paraffin method in plant cytology. *Stain Techn.*, 10, 95-6. 1935.

THE USE OF ACENAPHTHENE IN POLLEN TUBE TECHNIC

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Cambridge, Mass.*

ABSTRACT.—A procedure is described for growing pollen tubes in such a manner that a large number of clearly analyzed figures can be obtained. The pollen grains are sown on an artificial medium of sugar, agar, gelatin, and water, the proportions of each varying with the species of pollen grain used. The medium is smeared on the slide while still hot to insure a thin covering, and the pollen grains are dusted on when the medium has sufficiently cooled and hardened. The slides are placed in a staining dish provided with slide slots and a cover, the inside of the cover and the bottom of the dish being lined with moist, but not wet, filter paper. Acenaphthene crystals are lightly sprinkled on the bottom of the dish. The developing pollen tubes are thus exposed to the fumes given off by these crystals with consequent disturbance to the spindle mechanism. As a result, the chromosomes are not crowded on a metaphase plate but are widely separated in the tube facilitating any observations to be made.

The present technic was developed in order to facilitate an analysis of a large number of chromosomes in the pollen tubes of *Tradescantia L.* Previous technics were found inadequate because of the small number of suitable figures at any one time, and because the nature of the work demanded a considerable number of observations in order to arrive at a statistically significant analysis. Generally the chromosomes in passing down the tube are clumped in such a manner as to make observations difficult, if not impossible. A method was therefore sought whereby this objection might be remedied.

The method of growing the pollen tubes was essentially that described previously by Newcomer,¹ the medium consisting of 2 g. cane sugar, 0.5 g. agar, and 0.5 g. gelatin in 25 cc. of water. The sugar, agar and water were brought to a boil, and then as the medium cooled somewhat the gelatin was added slowly, stirring all the while to prevent clumping of the gelatin. The medium was smeared on the slides while still hot, two slides being prepared at a time. This procedure allows the first slide to cool sufficiently to permit sowing of the grains by the time the second is prepared. If the flowers are

¹Newcomer, Earl H. 1938. A procedure for growing, staining, and making permanent slides of pollen tubes. *Stain Techn.* 13, 89-91.

plentiful, a whole one per slide may be used by either dusting the pollen on or lightly brushing the opened anthers across the slide. If the number of flowers is limited, single anthers may be used. Clumping of the grains in any one spot results in poor orientation of the tubes, lessening the number of observations which might be made. Pollen grains buried in the medium do not grow as well as those resting on the surface. After sowing the grains, the slides are placed in a horizontal type staining dish. These dishes are provided with slots so that by placing the slides back to back it is possible to prepare 20 slides at once. The writer has taken precautions not to let the slides dry out while others were in the process of preparation, but in some instances the medium can become quite stiff and still give good germination. The staining dish was previously prepared by placing moist filter paper on the under side of the cover and on the bottom of the dish. It is very important that the paper be *moist, but not wet*, for



Fig. 1. A prophase in the pollen tube of *Tradescantia*.

excess water within the dish accumulates on the medium in droplets, causing reduced germination and poor development of the growing tubes. The staining dish may finally be placed in a moist chamber at room temperature as an additional precaution against drying out.

In order to obtain a large number of suitable figures it is desirable to stop the division of the generative nucleus at a time when the chromosomes are sufficiently contracted to permit an analysis, i.e., at metaphase. Colchicine was tried, with some success, but did not satisfy the demands of the experiment. Acenaphthene (naphthalene-ethylene) [C₁₀H₈(CH₂)₂] was suggested, and proved to be very satisfactory. Its action is similar to that of colchicine insofar as nuclear reaction is concerned. The spindle mechanism is definitely disturbed, the chromosomes merely dividing in place without moving to the poles. The solubility of acenaphthene in water is apparently very slight—chemical handbooks give no definite figures. With the crystals scattered on the bottom of the dish, its effectiveness appears to be dependent upon the penetration of the pollen tubes by the sub-

stance thru the medium of fumes. No attempt has been made to incorporate acenaphthene into the medium.²

Another advantage may be cited which makes the use of acenaphthene desirable. Usually the generative nucleus passes into the tube while in an early prophase stage, the nuclear membrane being still intact. Occasionally, in untreated material, the nucleus is retained in the pollen grain until a later stage, and when the chromosomes pass down, they do so in a sort of Indian-file fashion. Altho our knowledge of the action of acenaphthene on the nucleus is still in an experimental stage, there is definitely a greater number of tubes showing this peculiar chromosome arrangement in the treated material than in the untreated. The advantage of this phenomenon to clear observation and concise analysis of the individual chromosomes is obvious. Some slides will show over one hundred good figures.

The length of time needed for development of the nucleus to its metaphase stage limits somewhat the use of acenaphthene. Experi-



Fig. 2. The arrangement of the chromosomes in the pollen tube when subjected to the acenaphthene treatment.

ments with *Bellevalia Lapeyr.* proved its ineffectiveness to act in a short period of time, this genus requiring only $3\frac{1}{2}$ to 4 hours for the gametic division to be completed. *Tradescantia*, which requires 17-22 hours for development in artificial culture, responds very satisfactorily. Dahl (unpub.) has had similar good success with *Anemone* pollen which passes thru division about 38 hours after the time of sowing. Treated material is generally somewhat slower in development than that which is untreated.

Fixation of the pollen tubes in acetic alcohol (70 cc. of 100% ethyl alcohol to 30 cc. glacial acetic acid) gave beautiful preparations in some instances, but it was not always dependable. Belling's acetocarmine was used in staining. This method is by far the easiest and most rapid, and with firm pressure applied on the cover slip, a goodly number of figures can be obtained with all chromosomes on the same focal plane. For permanent slides, any of the standard fixatives and

²The acenaphthene used in this work is listed as 597, chemically pure, M.P. 93-94°, by the Research Laboratory, Eastman Kodak Company, Rochester, New York.

stains may be used. The use of orange G as a counterstain is undesirable since it is retained by the medium.

Growing pollen tubes in this manner likewise provides a convenient method of determining chromosome counts and chromosome morphology where other material such as root-tips and flower buds are not available. In some instances these pollen tube preparations are to be recommended over sectioned material and smears. Relational coiling can also be observed in the tubes to good advantage. This method implies, of course, using only pollen grains which can be grown on artificial media, and which have the gametic divisions in the tube and not in the grain.

POST-MORTEM AUTODIGESTION OF THE INTESTINAL MUCOSA OF THE TURKEY

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ABSTRACT.—A gradient of post-mortem activity exists in the small intestine of the turkey. It is manifested by immediate disturbance of intestinal epithelium of the duodenum, a somewhat similar but lessened effect in the jejunum, and a long delayed action in the ileum. The recognition of such a gradient stresses the rapidity with which one must proceed in handling such tissues for technical studies. In preparing intestinal tissues for histological examination, the duodenum and jejunum must be removed immediately after killing the specimen, opened, washed carefully with warm saline to eliminate excess debris, ferments and mucous, and fixed forthwith.

During the course of some histological work on the small intestine of the turkey, the author repeatedly obtained abnormal pictures of the mucosa from apparently normal birds. The material had been fixed by several methods: (1) dropping a small sector directly into the fixative, (2) opening and pinning before immersion in fixative and (3) filling a short length of intestinal tube with fixative and tying it off in sausage fashion. The interval before the fixative was actually applied to the tissue varied from 5–45 minutes after the bird had been killed. Such delay was necessary because samples of several tissues were being taken from each bird and some time was required to complete the manipulation. The duodenum in every case, upon sectioning, showed what appeared to be an immense amount of sloughing of the epithelium on the tips of the villi. This regular result was found less often in the jejunum and rarely in the ileum. Besides the sloughing of the epithelium at the tips, one frequently found that the epithelium of the body of the villus was separated from the membrana propria upon which it rested. This separation often continued into the crypts of Lieberkühn.

The foregoing conditions appeared so regularly that it was first considered to be "normal". One might assume that the rough type of chyme leaving the gizzard would tear off the villus tips. Rapid replacement of that epithelium would then ensue from the proliferating cells in the crypts. The presence of denuded villus cores with the consequent possibility of infections of various types seemed, how-

ever, to preclude this condition as a normal phenomenon in the living bird. In consequence, a faster and more refined method of fixation was tried.

Immediately after killing a bird, the abdomen was opened and a portion of duodenum removed, pinned open on a piece of cork, washed in normal saline at 39° C. and inverted in 10% neutral formol at the same temperature, the whole operation being completed within 5 minutes after the bird's head had been chopped off.

A second sample of duodenum was set aside, unopened, for 30 minutes before placing it, still unopened, in the warmed formol.

Samples of jejunum were fixed similarly within 10 minutes and after 30 minutes post mortem.

The accompanying photographs show the comparative results of rapid and delayed fixation on the four samples. Sectors opened, washed and fixed at once (Figs. 1, 2) show smooth contours on the villus plates, with the epithelium intact, while material from both

TABLE 1. EROSION OF VILLUS MUCOSA AT VARIOUS INTERVALS POST MORTEM

| Minutes after killing | Duodenum | | Jejunum | | Ileum | |
|-----------------------------|---------------|-----------------|---------------|-----------------|---------------|-----------------|
| | Fixed open | Fixed closed | Fixed open | Fixed closed | Fixed open | Fixed closed |
| 10 | (+) | ++ | - | (+) | - | - |
| 20 | ++ | ++ | - | (+) | - | - |
| 30 | ++ | ++ | (+) | + | - | - |
| 50 | ++ | ++ | (+) | + | - | - |
| 70 | ++ | ++ | + | + | - | - |
| 100 | ++ | ++ | ++ | ++ | - | - |

Explanation of Symbols:

-, no erosion

(+), villus tips show limited (or slight) desquamation

+, villus cores exposed

++, epithelium lost on most of basal part of villus—heavy erosion

the duodenum and jejunum which had not been washed and which were not immersed in fixative for the first 30 minutes post mortem (Figs. 4, 5) exhibit loss of the epithelium of the tips. The erosion is much greater in the duodenum than in the jejunum; in the latter some villi appear unaffected.

When these various samples were sectioned, those which had been washed and fixed quickly showed separation of epithelium from the membrana propria to be considerably less (Fig. 6), while in the un-washed, delayed samples there was extensive separation of epithelium along the sides of the villi, and sloughing of cells at the tips was pronounced (Fig. 7).

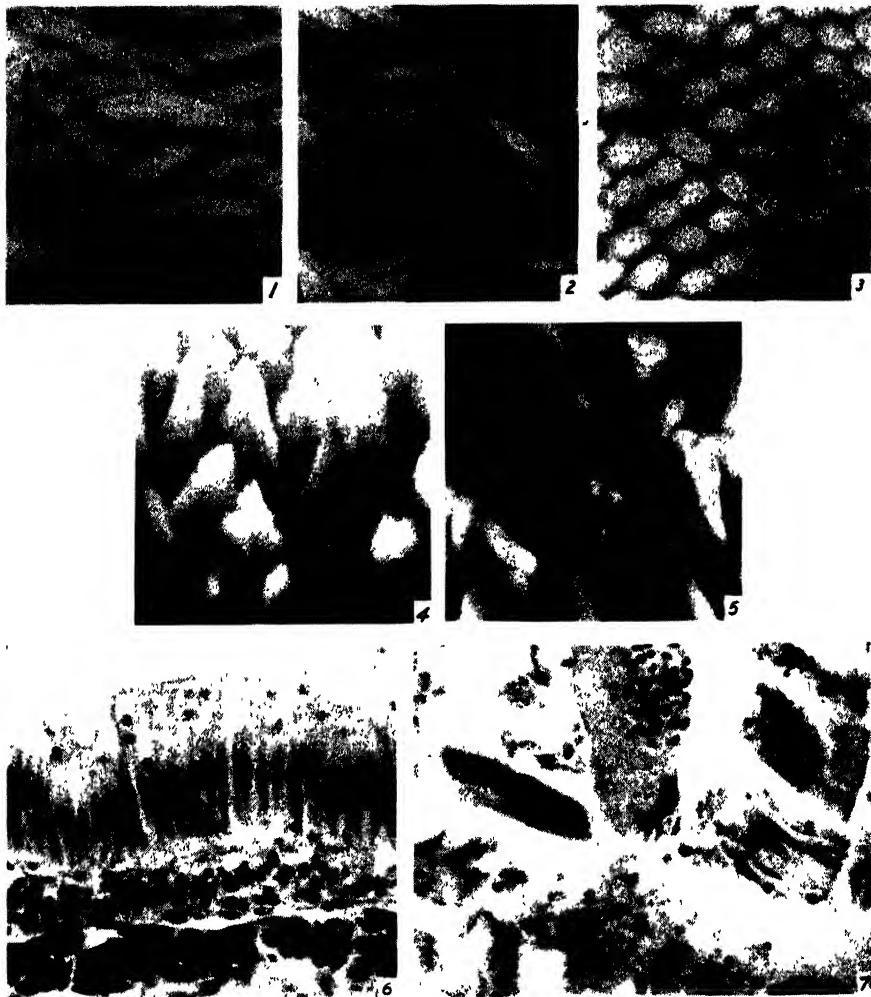


PLATE I

Figures 1, 2, and 3 were opened, washed and fixed flat within 10 minutes post mortem. Figures 4 and 5 were placed in fixative, unopened, 30 minutes post mortem and subsequently opened for photographing. The arrow indicates the antero-posterior axis of the intestine for Figures 1 to 5. Figures 1 to 5 are surface views taken thru water by reflected light. Note the difference in orientation of villus plates in the portions fixed flat, with those fixed closed. Sections were by the paraffin technic.

1. Duodenum, showing smooth contours of villus plates. $\times 14$.
2. Jejunum, the same. $\times 14$.
3. Ileum, as seen either after 10 minutes or 90 minutes post mortem. $\times 14$.
4. Duodenum. The villus tips are highly abnormal with considerable loss of epithelial cells. $\times 14$.
5. Jejunum. Only the very tips of the villus plates exhibit the desquamation of epithelium. $\times 14$.
6. Duodenal villus tip, fixed immediately, sectioned at 10μ and stained with Delafield's hematoxylin and eosin. $\times 384$.
7. Duodenal villus tip after delayed fixation. $\times 384$.

This technic of rapid versus delayed fixation was repeated on a second bird, all three portions of the small intestine being sampled. Three persons participated in the work so as to expedite the process and to make the results from the duodenum, jejunum, and ileum comparable. The findings from these latter preparations are summarized in Table 1.

Even the 10-minute sample of duodenum showed some slight loss of cells at the tips. This may have been due to the vigorous stream of warmed saline played upon the villi to remove debris, intestinal fermenta and mucous.

From the data in Table 1 it is evident that the duodenal epithelium is most susceptible to post-mortem damage, and that a decreasing gradient of susceptibility to erosion occurs from the duodenum to the ileum. The ileum is able to retain its epithelium intact even when the application of fixative is long delayed.

The differential in susceptibility of epithelium in the several parts of the small intestine may be associated with two factors. In the first place, the concentration of digestive fermenta is greatest in the upper part of the intestine. Secondly, the epithelium of the duodenum and upper jejunum is probably more loosely attached to the membrana propria than is that of the lower jejunum and the ileum; mitosis in the crypts of Lieberkühn indicates that epithelial replacement is greatest in the duodenum and diminishes thruout the remainder of the small intestine.

TIME SAVERS FOR FIXING AND DEHYDRATION

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ABSTRACT.—A double wash bottle for dispensing two-part fixing solutions is described. Equal volumes of each stock solution are delivered simultaneously into the same vial.

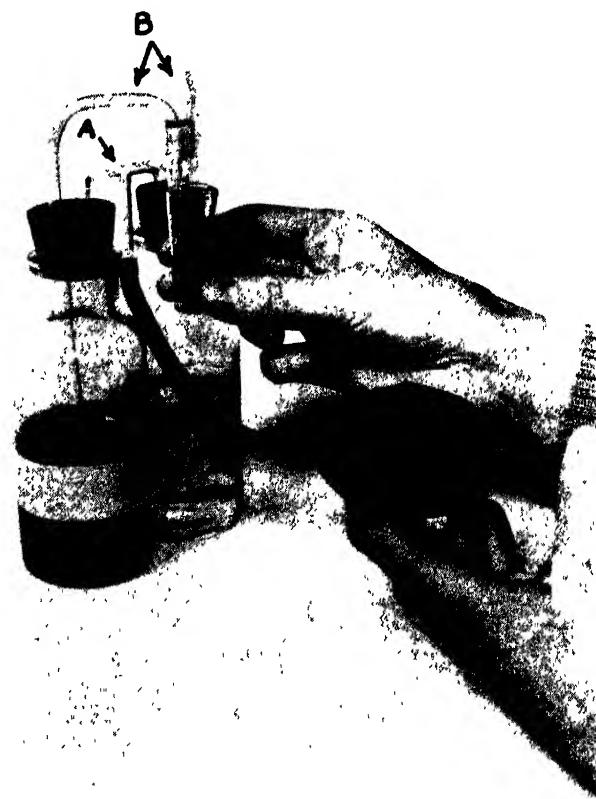


Fig. 1. Fixing-solution dispenser, showing use of thumb as valve when applying pressure.

A device for dehydrating tissues consists of a Buchner funnel closed at the bottom by a rubber tube and pinch clamp which facilitate changing the dehydrating alcohols. Tissues are placed in perforated brass baskets in the funnel.

¹Maintained in coöperation with Yale University, New Haven, Connecticut.

The time-consuming labor of routine fixing and dehydration operations may be reduced by the use of several simple devices.

Most two-part fixing-solution formulae can be so modified that equal volumes of each stock solution are used, and are readily dispensed from the double wash bottle shown in Fig. 1 which saves time in stopper pulling and pouring. The accuracy with which equal



Fig. 2. Dehydration apparatus with brass basket at upper right. The Buchner funnel is covered with a Petri dish.

volumes of the stock solutions are delivered is much greater than that secured by the usual estimates, and is dependent on filling the stock bottles to the same level and making both delivery tubes of the same dimensions thruout.

The fixing-solution dispenser is made with two wide-mouthed 8-ounce bottles stoppered with two-hole rubber stoppers. The ends of a glass T (Fig. 1, A) are bent downward (or upward if it is more convenient to have the bulb above the delivery tubes) and inserted

in one hole in each stopper. The delivery tubes (Fig. 1, B) which pass thru the other holes are bent as shown and the tips drawn out. There are no valves in the bulb which furnishes the pressure, the thumb serving as a valve when the solutions are to be dispensed. The bottles and the delivery tubes are taped together.

If the routine calls for the filling of numerous shell vials held in a block, the dispenser can be modified by lengthening the delivery tubes and supplying the air from above (ends of T bent upward) either by bulb or by mouth as with the common type of wash bottle.

When fixation is complete, the tissues are transferred from shell vials to soldered cylindrical baskets (Fig. 2) made of brass stock with about one hundred 0.5-mm. perforations per sq. cm. and 1/64 in. thick, with the smooth side turned inward to avoid catching and damaging the tissues. A convenient basket for root tips and other small materials is 12 mm. in diameter and 16 mm. in height. Few of even the finest root tips are lost thru the perforations of these baskets, particularly if longer tips are taken than is customary with other procedures.

Tissues are washed in the baskets, which may be corked to retain buoyant material. The baskets are then transferred to a 9-cm. Buchner funnel covered with a tight-fitting Petri dish (Fig. 2). The stem of the funnel is held in a ring-stand clamp and fitted with a short rubber tube closed by a pinch clamp. The dehydration series is run thru the material by pouring the solutions into the funnel with the pinch clamp closed and then drawing the solutions off from the bottom at the end of the proper time interval. The baskets are generally not corked during dehydration. An interval timer is excellent for keeping on schedule, thus effecting a further saving of time.

Infiltration is also accomplished in the baskets which are immersed in xylol and blotted with paper toweling to remove all paraffin from the perforations after the tissues have been removed. The funnel system of changing reagents should be applicable to the method of running up root tips cemented to cards.

Acknowledgments for some of the foregoing ideas are due to Dr. W. P. Stockwell, of the California Forest and Range Experiment Station, and to Mr. Ernest Jund, of the Division of Genetics, University of California, Berkeley, California.

DELAFIELD'S HEMATOXYLIN AND SAFRANIN FOR STAINING PLANT MATERIALS

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ABSTRACT.—An improved schedule is suggested for staining plant materials in Delafield's hematoxylin and safranin. Tissues are stained first in Delafield's hematoxylin. A short bath in acidulated water (1 or 2 drops concentrated HCl to 100 cc.) removes objectionable precipitates, and at the same time serves as a destaining agent. The acid bath must be followed quickly by a thoro wash in tap water, or dilute lithium carbonate solution, to restore the original dark blue color (made reddish in the acid bath) of the hematoxylin and to "set" the stain. Once the hematoxylin solution is satisfactory, none of the reagents ordinarily used will remove it—unless they contain acid. Tissues are counterstained in rapid safranin (5 drops analin in 100 cc. of 1% safranin O in 50% ethyl alcohol); this materially lessens the time necessary for staining. The safranin is destained in 50% ethyl alcohol (which does not affect the hematoxylin) until sharp differentiation is secured. If destaining is too slow, or differentiation poor, a quick rinse in acidulated 50% alcohol usually sharpens contrast of the stains. This must be followed quickly by a wash in 50% alcohol containing lithium carbonate to neutralize the acid. Dehydrate, and mount as usual. This schedule allows each stain to be individually, and independently, controlled at the will of the operator.

Delafield's hematoxylin is one of the most useful histological stains for the precise staining of cellulose cell walls. Methods of using this stain, however, have often yielded uncertain results, varying with the skill or luck of the technician. Delafield's hematoxylin should stain well, when used alone, except for characteristic precipitates that often appear in the tissues. An excellent double stain is Delafield's hematoxylin and safranin, but frequently this combination appears to cause trouble. Sharp differentiation is not always obtained, and objectionable precipitates often remain in the tissues after staining. The addition of safranin may unduly mask the hematoxylin in the tissues and cause a muddy stain. Staining may be a lengthy process with uncertain results in the finished slides. The difficulty in getting uniformly good results has caused many workers to dislike, or reject, this combination of stains. Nevertheless, when a well-stained slide is produced, these stains appear to justify the claims made for them.

Schedules for Delafield's hematoxylin and safranin ordinarily recommend that tissues be stained in safranin first, at least several hours, and sometimes over night or longer. Some older schedules even suggest boiling freehand sections in safranin. After staining in safranin, the tissues are destained in 50% ethyl alcohol (sometimes acidulated) almost to the proper color; then Delafield's hematoxylin is applied. Within a few minutes the hematoxylin is usually dark enough, and a bath in acidulated water or alcohol is then used to differentiate the stains. The acid removes both stains from the tissues. Consequently, the interval allowed in each stain and the duration of the acid bath must be carefully timed or poorly differentiated tissues result. Obviously, the quality of the finished slide cannot be judged properly until after the final acid bath; if the stains are not then satisfactory, the whole process must be repeated—usually after decolorizing the tissues in acidulated water or alcohol.

When Delafield's hematoxylin is used in combination with eosin or erythrosin, however, the above procedure is commonly reversed; the tissues are first stained in the hematoxylin, then in the counterstain. Chamberlain¹ indirectly indicated that safranin could be used as a counterstain when he stated: "After the hematoxylin is just right, apply a contrast stain, if you wish to double stain." Later, however, he gave detailed directions (pp. 88-91) in which he recommended safranin first, followed by Delafield's hematoxylin. Recently, in staining meristematic tissues, Boke² used safranin dissolved in xylol as a counterstain following Delafield's hematoxylin.

The possibility that safranin could be used profitably as a counterstain *following* Delafield's hematoxylin, in the routine staining of plant materials, was suggested to the writer in 1931 by Professor R. B. Wylie; and experiments along this line were undertaken, using the following solutions:

Delafield's hematoxylin, made according to the formula of Stirling and Lee as given in Chamberlain (1932). Only fully aged, naturally ripened, stain was employed. Coleman and Bell hematoxylin was used, Cert. Nos. FH-5, and FH-11.

Rapid safranin, made by adding 5 drops of anilin to 100 cc. of a 1% solution of safranin O in 50% ethyl alcohol. The anilin acts as a mordant. National Aniline safranin O was used, Cert. Nos. NS-10 (dye content 94%) and NS-9 (dye content 97%).

Points favoring the use of these stains in this order may be briefly summarized as follows: (1) With the hematoxylin applied first, it is easier to get the stain just the shade of color desired. The bath in

¹Chamberlain, C. J. 1932. Methods in Plant Histology, 5th Ed. University of Chicago Press. See p. 51.

²Boke, Norman H. 1939. Delafield's hematoxylin and safranin for staining meristematic tissues. *Stain Techn.*, 14, 129-31.

acidulated water following the stain dissolves precipitates that so often accompany Delafield's hematoxylin, and also serves as a de-staining agent. Once the hematoxylin is "set" in the tissues, none of the reagents ordinarily used will decolorize it, unless they contain acid. (2) When differentiating in 50% ethyl alcohol after counter-staining in rapid safranin, the counterstain is washed from cellulose cell walls faster than it is from lignified structures. Since this treatment affects only the safranin, it can be continued until the blue of the hematoxylin stands out in sharp contrast to the red of the safranin. Each stain may thus be individually controlled at the will of the operator. The use of rapid safranin greatly shortens the time of staining. Good results can be obtained with the usual safranin solutions, but the time of staining must be considerably increased.

The time of staining must be determined experimentally for various tissues. Ten minutes in Delafield's hematoxylin followed by a 15-minute stain in rapid safranin has been about right for sections of *Osmunda* rhizome killed in a mixture of formalin, alcohol and acetic acid (denoted, "F. A. A."). With most leaf sections, a 5-15 minute stain in Delafield's followed by 10-15 minutes in rapid safranin should give good results. Only tissues extremely difficult to stain should take more than 30 minutes in the hematoxylin. Tissues often stain poorly, or not at all, if the killing fluid has not been thoroly washed out before embedding. Delafield's hematoxylin stains especially well following killing fluids containing chromic acid, but a satisfactory stain can be obtained following practically any of the usual killing agents. If tissues are difficult to stain, a 5-minute bath in 1% aqueous potassium permanganate is suggested as a mordant.

The accompanying provisional schedule has been used with success for the past 7 years in classes in histological technic, and has proved effective for critical research materials, especially when careful measurements must be made of plant cell walls. Tissues stained in Delafield's hematoxylin and safranin are excellent for photographic purposes. The accompanying schedule, written for paraffin sections, may easily be modified for use with free-hand sections or bulk material.

STAINING SCHEDULE FOR DELAFIELD'S HEMATOXYLIN AND SAFRANIN

1. Remove paraffin and get slides into tap water. All alcohol should be removed from tissues or staining may be spotty.
2. Stain in Delafield's hematoxylin—5 minutes to 1 hour. As staining is progressive, try minimum time first, then increase by multiples of 5 minutes—up to 30 minutes. If tissues are unaffected

after 30 minutes, continue staining for a total of one hour. If tissues are unstained after one hour, look for causes of failure other than the hematoxylin.

3. Rinse well in tap water to remove surplus stain.
4. Examine under the microscope. Sections at this point should be somewhat overstained. Destain in acidulated tap water (1 or 2 drops concentrated hydrochloric acid in each 100 cc. water). Gently agitate slides in acidulated water until sections assume a reddish color—usually in 3–10 seconds. Time in acidulated water must be varied according to density of stain desired, type of tissue, and the concentration of acid used. The acid destains the tissues, and dissolves objectionable precipitates. Because these precipitates so frequently occur, the acid bath should always be used. (If the color is too light, thoroly wash out all acid in tap water and restain in the hematoxylin.)
5. Wash *immediately* in clean tap water. Allow to stand in several changes of tap water until sections assume their original dark blue color. If the blue color does not soon reappear, or if a more positive method is desired, allow slides to stand in 0.1% aqueous lithium carbonate until the color is restored. Five minutes should be sufficient. The carbonate provides the necessary alkaline medium for the hematoxylin, and often intensifies the stain. Use a weaker carbonate solution if the sections persistently assume a hazy, light blue color. The lithium carbonate solution may macerate certain soft tissues. If this occurs: (1) add 5 or 10 drops of 0.1% lithium carbonate solution to a Coplin jar of tap water, (2) add 3–4 drops of 5% ammonia to the tap water, or, (3) use plain tap water (usually slightly alkaline). The color is usually darker when lithium carbonate is used than it is when plain tap water is employed.
6. Rinse in tap water to remove carbonate solution. Examine under the microscope and repeat steps 2, 3, 4, 5, and 6, if necessary, to get proper density of stain. If color is too light, either shorten the time in acidulated water, use less acid, or stain longer.
7. Pass slides thru alcohol series to 50% alcohol.
8. Stain in rapid safranin—5 minutes to over night. Try minimum time first, then increase in multiples of 5 minutes—up to 30 minutes. Usually, difficult tissues will stain in 1–4 hours; rarely should it be necessary to stain over night.
9. Drain, blot, or rinse off (with slightly used or clean 50% ethyl alcohol) the excess safranin, and differentiate in clean 50% alcohol. This destains the safranin only, and should be continued until sharp differentiation is obtained.

10. If destaining of safranin is too slow, or differentiation poor, dip and gently agitate the slide in acidulated 50% alcohol (1 or 2 drops hydrochloric acid in 100 cc. alcohol) for a few seconds. This quickly removes the excess safranin, and slightly reduces the hematoxylin, but usually sharpens contrast between the stains. Repeat if necessary.

11. Wash *immediately* in clean 50% alcohol made alkaline by adding 5-10 drops of 0.1% aqueous lithium carbonate to a Coplin jar of the alcohol. Allow slides to stand in fresh, alkaline, 50% alcohol for at least 10 minutes before dehydrating.

12. Dehydrate, clear, and mount in neutral balsam.

Note: To stain in Delafield's hematoxylin only, omit steps 8, 9, 10, and 11.

Whenever a metallic-appearing scum forms on the surface of the hematoxylin it should be filtered, or skimmed off with the torn edge of a piece of filter paper. Delafield's hematoxylin may be used full strength or, preferably, diluted with distilled water. The writer ordinarily dilutes the hematoxylin with one-third its volume of distilled water. Greater dilutions may be used, usually with more precise results, but the time of staining must be increased accordingly. Dilution of the safranin with 50% alcohol may be necessary for certain tissues, but, as with the hematoxylin, the staining is often more precise. A few experiments will determine the best procedure for each lot of new material.

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A SIMPLE STAINING METHOD FOR HISTOLOGY AND CYTOLOGY

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In 1937 Cannon¹ called attention to the possible histological uses of a greenish-black dye put out by the British Dyestuffs Corporation under the name chlorazol black E. This proves to be an acid dye of the azo group, (Colour Index No. 581), a sulfonated triazo dye, which has been sold by various manufacturers under a variety of names. The National Aniline and Chemical Co., upon request to furnish a sample of this dye, have supplied the product which they sell under the name Erie black GXOO; I. E. Pont deNemours and Co. in response to a similar request furnished a sample labeled pontamine black E. These two samples have been submitted to spectrophotometric analysis and appear to be almost identical, altho Erie black GXOO is slightly bluer than pontamine black E.

Apparently this dye should be better known. The writer has tested 1% solutions of both samples in 70% alcohol, freshly prepared and unfiltered, on histological and cytological material, and has found them to give excellent pictures, both samples responding similarly. The method of staining is as follows:

Run paraffin sections down thru water. Stain 5-10 minutes (rather than 15-30 minutes as suggested by Cannon) in a 1% solution in 70% alcohol, freshly prepared and unfiltered.² (Cannon calls for a saturated solution of dye in 70% alcohol). Drain off excess dye, wash in 95% alcohol, absolute alcohol, xylene, and mount. No mordant and no differentiation are required.

In the epithelium tissue of a rabbit embryo (Zenker's fixation) the cells were definitely outlined black; the chromatin, dark; cytoplasm, greenish gray; and the nucleoli, black. The muscle fibers and lymphocytes were a decided black, and the blood cells, a yellowish green.

Zenker-fixed kidney sections and Bouin-fixed sections of intestine stained in varying shades of green, gray and black, with the blood cells of the kidney sections a light green.

Sections of *Puccinia* on aster (fixed in Allen's B-15 fluid) showed a jet black outline of the cell walls; the cytoplasm and plastids, grayish

¹Cannon, H. J. A new biological stain for general purposes. *Nature*, **139**, 549. 1937.

²In the present work staining periods of 1, 5, 10, and 15 minutes were tried.

green; and the nucleoli, dark green. The infected part and the hyphae stained yellowish green.

A cross section of a fern leaf (Flemming fixation) stained the various structures as follows: cell walls a definite black; the epidermis walls, heavy black; nuclei green, with nucleoli a dark green; cytoplasm light amber; plastids gray. The suberized walls of midrib and veins were dark amber.

In sections of an onion root tip (Flemming fixation), the cell walls stained dark gray almost black; cytoplasm, grayish green; nucleus a yellowish green, with the nucleoli a deep yellow (amber).

This stain certainly shows sharp differentiation for either histological or cytological purposes, and merits wider use for general purposes.

CHLORAZOL BLACK E AS AN ACETO-CARMINE AUXILIARY STAIN

B. R. NEBEL, *Geneva, N. Y.*¹

ABSTRACT.—In making chromosome counts on plants and plant parts treated with colchicine it was found that in cases where aceto-carmine alone is not satisfactory—as in axillary buds of apple, pear, plum, peach, apricot, and cherry—the following method was effective: Dissect out the meristematic parts of the axillary bud under a binocular (or cut free-hand sections) and transfer the dissected tissue immediately to a solution of 3 volumes alcohol to 1 volume acetic acid for killing and fixing. Let the fixative act at least 10 minutes; a longer time, 12-24 hours, improves the staining quality. Wash in at least 3 changes of 70% alcohol to remove most of the acid. Stain for 5-25 minutes in 1% chlorazol black E² in 70% alcohol. Rinse in 3 changes of 70% alcohol to remove excess stain. Transfer the material to a slide, cover with a drop of aceto-carmine, and if necessary, dissect further under a binocular. Cover with cover glass, heat, flatten and seal, or run Zirkle's fluid under the cover for permanent mounting. For smears of sporocytes, chlorazol black E may also be employed alone, or in combination with aceto-carmine, if a dark purple nuclear stain is desired.

The inducing of polyploidy in deciduous fruit trees and herbaceous plants by the use of colchicine and other chemicals depends to a large extent on valid methods of tracing the progressive action of the drug in the tissue and later in detecting tetraploid tissue areas on diploid plants. In deciduous fruits various concentrations of colchicine in 5% alcohol were applied to axillary buds. The penetration of the drug into the meristems of the bud and its action thereupon were followed by successive dissection of treated buds. The success of the treatment was gauged by dissecting axillary buds laid down 1-3 months after the first treatment, that is, after growth and shoot development had occurred from the bud first treated.

¹Approved by the Director of the New York Agricultural Experiment Station as Journal Paper 349. Dec. 7, 1939.

²Attention was called to this dye by the Biological Stain Commission; see preceding article by Daigrow. The sample used in this work was obtained from The National Aniline and Chemical Co. under the name Erie black GXOO; the DuPont Co. uses the name pontamine black E. All three of these names are regarded as virtual synonyms by the Stain Commission. Since the work was completed, this dye has been put on the certification basis by the Commission.

In vegetative tissue, nuclear stages are difficult to observe and chromosome plates were not readily stained with aceto-carmine in deciduous fruits. The following method was found useful to overcome this difficulty.

Axillary buds were prepared for staining by cutting from the main stem or branch as in nursery budding. Such buds may be transported in moist dishes and kept for one hour without the loss of mitotic cells in the meristems. The use of crushed ice during transport and storage is advisable.

The following procedure may be modified to satisfy individual needs: An apple bud, for example, was placed in a drop of water on the stage of a binocular, dissected from its woody base, then peeled to free the central axis or axes carrying the meristematic leaf and shoot

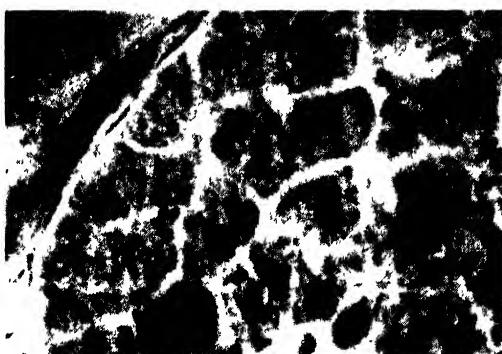


Fig. 1. Tissue of a pear bud treated with colchicine for 5 days with 3 daily applications and then allowed to recover for 2 days.

initials. When only the desired parts remained, the axis or axes were cut longitudinally and quickly immersed in 3:1 alcohol and acetic acid for killing and fixing. Better differentiation and more contrasty staining were achieved with material which remained in the fixative over night, than with fixation for 5-10 minutes only. When speed was essential, routine results were obtained with short fixation. After fixing, the material was transferred to three changes of 70% alcohol to remove the acid from the tissue. It was found inadvisable to cut the total time in 70% alcohol to less than 5 minutes.

The stain was prepared by dissolving 1% by weight in 70% alcohol and filtering thru a coarse filter. The stain is somewhat colloidal and tends to clog a fine filter. Small brushes were used for transferring the material. The exact time for staining had to be tested for each species of plant; in general, it should not be less than 5 minutes or more than 25 minutes.

The period of staining was followed by rinsing in three changes of 70% alcohol. The material was then transferred to a slide carrying a small drop of aceto-carmine, and all non-meristematic parts were



Figs. 2-5. Sporocytes of *Tradescantia reflexa* in first meiotic division. All exposures were identical, on Wratten M blue label plates developed at 65° for 12 minutes in D76; and printing was identical in all cases.

Fig. 2. Aceto-carmine after fixation in 3:1 alcohol-acetic-acid.

Fig. 3. Carmine, followed by chlorazol black E; mounted in Zirkle.

Fig. 4. Carmine, and chlorazol black E; mounted in aceto-carmine.

Fig. 5. Chlorazol black E only; mounted in 45% acetic acid.

carefully dissected away under a binocular. With the smallest amount of material finally remaining on the slide, results were found most satisfactory. The preparation was then covered, heated, lightly

squeezed and examined. If permanent mounts were desired, the McClintock technic was used or the aceto-carmine was gradually replaced by Zirkle's fluid.

The visual impression obtained from examining the slide before applying the aceto-carmine was that no appreciable amount of stain had been taken up by the chromatin. However, after the use of both stains, the chromosomes are stained sharply as they would not be with aceto-carmine alone. This is illustrated in figure 1 representing pear tissue.

While, in the present investigation, chlorazol black E was thus used primarily to stain tissues in which chromatin could previously not be stained quickly, a series of slides was made to test the new stain on *Tradescantia* sporocytes which have a high nuclear dye affinity.

The anther content was smeared on a clean slide and covered with a large drop of alcoholic acetic acid (1 volume glacial acetic to 3 volumes alcohol). After 1 minute, the slides were transferred to Coplin jars containing 70% alcohol. From there on the slides were treated differentially as illustrated in figures 2 to 5. Figure 2 is the check, the material being stained and mounted in aceto-carmine. This resulted in high transparency with a high density range within the chromosomes which is often confusing to the eye. When chlorazol black E was used after carmine and the material was then mounted in Zirkle's fluid, the density of the chromatin stain was intermediate and well adapted to visual work (Fig. 3). In this preparation the cells were neither swollen nor flattened. The material in Fig. 4 was treated with carmine before and after staining with chlorazol black E, and was mounted in carmine. The swelling was intermediate and the cells were flattened in this procedure. The chromatin was densely stained.

Finally chlorazol black E was used alone and the material mounted in clear 45% acetic acid without carmine. The chromatin stain was very dense and well differentiated (Fig. 5). The cells were much flattened.

Thru the courtesy of Mr. J. D. Nantz of the National Aniline and Chemical Co., two black purified derivatives of chlorazol black E were used on *Tradescantia* sporocytes. Both of these stained nuclear material after acid fixation only a weak gray so that they appear to have no value for the present purpose. The names of these dyes were: Buffalo black NBR and durol black 2 B.

In conclusion: The ease of applying chlorazol black E, its high nuclear affinity and the fact that it appears relatively stable in acid solution commend the stain for general use.

A TECHNIC FOR STAINING MOUSE PITUITARY

EARL B. SCOTT, *Department of Biology, University of Pittsburgh*

Many technics for histological staining of the pituitary gland have been devised. Since most of the methods described have been for animals other than the mouse, and since the mouse hypophysis, for some unknown reason, is difficult to stain by the accepted technics, the present writer has worked out a modification of Mallory's triple staining method.

Removal of the Pituitary. After the skull cap has been removed, the brain is loosened at the anterior end and carefully reflected back. The hypophysis is now exposed. The point of a wet needle is then used to loosen the capsule of the gland, which comes away easily, and a wet scalpel is slid under the pituitary. In this manner the gland can be easily lifted from its shallow sella. Sometimes the hypophysis folds itself double on the scalpel, but it can be very easily straightened out with a wet needle. It is important that the needles and scalpel be wet.

Fixation and Embedding. The pituitary is fixed in Zenker-formol for 24 hours and washed in running water for 24 hours. The tissue is then dehydrated in two changes of cellosolve¹ and infiltrated and embedded in Hance's rubber paraffin.²

Staining. The following steps are used in staining:

Xylol; cellosolve; cellosolve-iodine solution, 10 min.; cellosolve; distilled water, (two changes); Mallory's 0.5% acid fuchsin,³ 5 min.; distilled water (two changes); 15 min. in Mallory's Solution 2 (aniline blue,³ 0.5 gm.; orange G, 2.0 gm.; 1% phosphomolybdic acid, 100 cc.); distilled water (two changes); cellosolve (a dip or two); distilled water; Mallory's Solution 2, 1½ min.; distilled water (two changes); cellosolve (two rapid changes); oil of thyme and cloves; xylol.

By using the above method, acidophiles were stained a brilliant red, basophiles blue, and the chromophobes were unstained. The same method was used in staining cat hypophyses with excellent results. In the cat, negative images of the Golgi apparatus were evident.

¹Inkster, R. G. The use of 'Cellosolve' for rapid dehydration in paraffin embedding and in staining of sections. *J. Path. and Bact.*, **44**, 269. 1937.

²Hance, R. T. A new paraffin embedding mixture. *Science*, **72**, 253. 1933.

³Coleman and Bell dyes were used by the writer in this technic.



LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

The abstracts given here are intended primarily for laboratory use; consequently the technic in each instance is given in as much detail as possible.

J. A. de Tomasi

Abstract Editor

MICROSCOPE AND OTHER APPARATUS

FEDER, J. M. *Adaptation of the rolls razor to a new type of microtome blade.*
J. Lab. & Clin. Med., 25, 202-4. 1939.

The blade of the rolls razor is made of high quality English steel. It is hollow ground and readily honed and stropped. To adapt it for use on a microtome, a holder made from a piece of tool steel approximately the size and shape of a microtome blade (12.5 cm. long and 0.5 cm. thick) is recommended. The holder is 2.9 cm. wide, and at the thinnest portion 0.2 cm. thick, being tapered to a blunt edge. In the center of this bar, a slot 3.8 cm. long and 1.5 cm. deep is cut to hold the blade. On the left end of this slot, two pins engage in the two depressions on one end of a rolls razor. The opposite end of the blade is fitted with an inset steel ball. A set screw with a knurled head is placed in the right end of the holder and so arranged that when tightened its inner edge engages the ball of the blade making it immovable. The blade is placed in the depression so that the two small pins engage in the proper depressions and the set screw is tightened. The blade proves to be safe, efficient and economical.—*John T. Myers*.

HERCIK, FERDINAND. *Die Fluoreszenzmikroskopische Analyse der α -Strahlenwirkung.* *Protoplasma*, 32, 527-35. 1939.

For studies of the effect of alpha-radiation the author takes advantage of the fact that epidermal cells of *Allium cepa* become fluorescent, without any sign of injury, when immersed in an aq. solution (1:1000 dist. water kept at pH 5.8 with HCl) of potassium fluorescein (uranin) as determined by Strugger (*Flora*, 132, 253, 1938).

Within 2-5 min. the healthy cells give a beautiful yellow green fluorescence by ultraviolet light. This first appears in the cell walls, then disappears to appear in the protoplasm and cell nuclei. The source of the alpha-radiation is a polonium preparation, the strength of which is previously determined by means of a Geiger counter. When tissues previously exposed to the radiation are immersed in the solution of fluorescein, the fluorescence appears in varying degrees, the blue-green illumination appearing, in some cells, only in the protoplasm, and in others in the vacuolar sap with varying intensities. The author claims the degree of fluorescence to be a far more delicate indication of damage to cells than the staining reaction with erythrosin.—*Robert Chambers*.

NEUWEILER, N. G. *Darkground illumination and Rheinberg colour discs—Some new and simple ideas.* *The Microscope*, 13, 81-2. 1939.

The simplest method of securing satisfactory darkground illumination is to insert suitable stops in the stop-holder of an Abbé condenser. This scheme works well at low and medium magnifications, but with a high-power objective some glare appears. The difficulty may be met by inserting the stops between first and second lenses of the condenser. Rheinberg color discs, which produce color effects with many microscopic objects, may be similarly inserted into the condenser.—*C. E. Allen*.

PIEKARSKI, G., und RUSKA, H. Über mikroskopische Untersuchungen an Bakterien unter besonderer Berücksichtigung der sogenannten Nucleoide. *Arch. Mikrob.*, 10, 302-21. 1939.

By means of the electron microscope (described by: E. Ruska, *Zts. f. Phys.*, 87, 580, 1934; B. v. Borries and E. Ruska, *Wiss. Veröff. Siemenswerke*, 17, 99, 1938) exceptionally fine photographs were obtained of various bacteria at magnifications ranging from 5,000 to 18,000 diameters.—*Merritt N. Pope*.

MICROTECHNIC IN GENERAL

(ANONYMOUS). Nevillite V. Perfect substitute for balsam. *El Palo Alto News*, 4, 10-1. 1939.

Canada balsam mounts become yellow upon aging and turn decidedly acid in less than a year. A new synthetic resin, nevillite V, appears to answer the requirement of technicians for a thoroly reliable mounting medium. Nevillite V is a naphthene polymer, a cycloparaffin, available in the form of colorless clear lumps. Its outstanding characteristics are: it is inert, homogeneous, resistant to the action of light, also of dilute acids and alkalies; its acid number is negligible; M. P. 155° C.; it is soluble in hydrocarbons, including paraffin; it is insoluble in water, and alcohols. Toluene appears to be the most satisfactory solvent. For general purposes one should use a solution of 60% by weight, which yields a thinner fluid than the average balsam-xylene medium. The advantages of using nevillite V are that there is less solvent to evaporate, coverslips are more easily applied, and there is far less chance of trapping air bubbles in the mount.—*J. A. de Tomasi*.

ARMITAGE, F. D. Dioxan in microscopical technique. *The Microscope*, 3, 212-6. 1939.

Dioxan is recommended in the preparation of fixatives. Tissues fixed in reagents insoluble or slightly soluble in dioxan should be well washed in water. Those fixed in readily soluble reagents can be washed immediately in dioxan. The following schedule is recommended for mammalian testes: Fix in Bouin's solution, 12-24 hr.; wash in 3 successive lots of 100% dioxan, 1 hr. each; change to fresh dioxan, and leave until required; place in equal parts dioxan and paraffin (m.p. 52°), 2-4 hr.; warm gently; shake gently; place in second dioxan-paraffin bath, 1 hr.; pure paraffin, 2-4 hr.; fresh paraffin, 1 hr.; imbed.

For mounting, either Canada balsam or the following dioxan-camsal-sandarac medium is satisfactory: Dissolve 25 g. gum sandarac in excess of dioxan; filter thru glass wool; evaporate filtrate to thick syrup, preferably on an oil bath; rub together in a mortar 3 g. salol and 2 g. camphor, heating gently to liquefy if necessary; add 1 cc. of this "camsal" to each 20 cc. of sandarac syrup; evaporate, if necessary, to the usual xylol-balsam consistency.—*C. E. Allen*.

CARLETON, H. M., and LEACH, E. H. An improved method for flattening out paraffin sections. *J. Path. & Bact.*, 49, 572-6. 1939.

A method is proposed which is claimed to be particularly valuable when fixation has been faulty or post mortem changes are present. Sections prepared by this technic are superior to controls flattened on water, and closely approximate conditions in living cells and tissues. The cast block must be water-free. Sections are treated as follows: Put a small drop of diacetin (glycerol diacetate) on a clean slide. Do not albuminize the slide. Spread the drop with a section-lifter over an area at least as big as the flattened section will be. Put the section on the diacetin, place slide on hot plate, and help section flatten with aid of needles. Wipe off excess diacetin. Stand vertically in drying oven at 37° C., or, better, at 50°. After a few minutes more diacetin can be wiped off. After 12 hr., or preferably 1-2 days, remove and allow to cool. Sections stored in an oven for a few weeks become diacetin-free and damaged. Add xylene. After removal of all paraffin, add 0.1% celloidin in equal parts abs. alcohol and ether. After wiping off excess, allow celloidin to set, but not to dry completely (10-20 sec.). Plunge into 90% alcohol and leave for few minutes. Run down to water, stain as desired, dehydrate in graded alcohols. As celloidin is soluble in abs. alcohol, use 96% alcohol for differentiation. Place in abs. alcohol the shortest time possible

for dehydration or sufficient removal of celloidin; lower gently into xylene and leave 2 min. undisturbed; mount.—*S. H. Hutner.*

COLE, W. C., and SMITH, F. R. A microscopic technique for studying fat globules in dairy products and other oil in water emulsions. *J. Dairy Science*, 22, 420-1. 1939.

The method used for examining fat globules in milk, cream, chocolate milk, ice cream mix, evaporated milk and mayonnaise is: Dilute the material to be examined in dist. water (usually 1 to 100). Place approximately 0.02 ml. on a clean slide. Add about 0.01 ml. of an alkaline aq. solution of 1% nile blue sulfate (NaHCO_3 to make alkaline), and mix well. Place cover slip over mixture, and examine under microscope.—*H. Macy.*

DERBY, J. T. A substitute for ethyl alcohol. *The Microscope*, 3, 243-6. 1939.

Cellosolve (ethylene glycol mono-ethyl ether) is suggested as a substitute for ethyl alcohol. The following fixing-imbedding schedule is recommended for plant tissues:

After fixing and washing in water for 12 hr., dehydrate in cellosolve in the following dilutions (aq. solutions), 3 changes per day: 15%, 35%, 50%, 65%, 75%, 85%, 95%, 100% (change the 100% once). For the finest cytological details, a closer series may be desirable. Clear in xylene in the following combinations: 25% xylene, 75% cellosolve; 50% xylene, 50% cellosolve; 75% xylene, 25% cellosolve; 100% xylene (change the 100% once). Infiltrate with paraffin. Imbed.

Cellosolve may also be used instead of abs. alcohol in preparing material for venetian turpentine mounts.—*C. E. Allen.*

EARL, W. R. Iron hematoxylin stain containing high concentration of ferrous iron. *Science*, 89, 323-4. 1939.

A useful modification of Janssens' iron hematoxylin stain is suggested. Instead of the ferric salt, a mixture of equal parts of ferric and ferrous ammonium sulfate is recommended as follows: $\text{Fe}_2(\text{SO}_4)_3(\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$, 20 g.; $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 20 g.; hematoxylin dissolved in 25 cc. abs. methyl alcohol, 1 g.; glycerin, 25 cc.; water, 100 cc. Sections and whole mounts of tissue cultures show a transparent blue nuclear stain and require no differentiation other than soaking for a few minutes in a number of changes of dist. water, or in VanGieson piero-fuchsin for counterstaining.—*J. A. de Tomasi.*

KIRKPATRICK, J. and LENDRUM, A. C. A mounting medium for microscopical preparations giving good preservation of colour. *J. Path. & Bact.*, 49, 592-4. 1939.

An experimental study revealed that the fading of delicate dyes was correlated with the rapid acidification of the Canada balsam used. A study of a large number of natural and synthetic resins revealed that "distrene 80", a polystyrene of molecular weight approximately 80,000, obtained from the Messrs. Honeywill and Stein of London, was superior to any of the others. It dissolves in xylene; but as this solution in drying retracts badly under the coverslip, it is recommended that 7.5 cc. of tricresylphosphate be added to 40 cc. of xylene. In this quantity of the solvent 10 g. "distrene 80" should be dissolved. No acidification and no fading have been noticed with this mounting medium; it is cheaper than Canada balsam, and its melting point is much higher.—*S. H. Hutner.*

LAPIN, W. K. [On the possibility of replacing Oleum caryophyllum in cytological practice with some other essential oils]. *Works All-Union Scient. Res. Inst. Humid Subtropics*, 1, Series 4, 75-8. 1937.

In searching for a substitute for clove oil as a differentiating agent in the Newton's gentian violet method, twelve different essential oils produced in Russia were tested on sections from root tips of *Poncirus trifoliata*, *Ficus carica*, and *Zea mays*. Imported clove oil (no source indicated) served as a control. With materials requiring a normal differentiation, best results were obtained by using the oils extracted from *Andropogon citratus*, *Manardia* and *Citrus salicifolia*. Immediate differentiation was obtained with oils from *Lavandula spica*, *Lavandula lanata* and *Citrus bigaradia*.—*J. A. de Tomasi.*

LILLIE, R. D., and EARLE, W. R. Iron hematoxylins containing ferric and ferrous iron. *Amer. J. Path.*, 15, 765-70. 1939.

Studies of Janssens' iron hematoxylin (Stain Techn., 14, 53-4, 1939) were extended to include the effect of ferrous iron on its staining and keeping qualities. Solutions which were made up with a high concentration of FeSO_4 remained usable for about 1 year while those without any, or with low concentration, spoiled in a few hours. The following staining solutions are recommended: (A) $\text{FeNH}_4(\text{SO}_4)_2$ (violet crystals), 15 g.; FeSO_4 , 15 g.; dist. water, 100 cc. (B) Hematoxylin, 1 g.; alcohol (95%), 50 cc.; glycerol, C.P., 50 cc. Mix A and B in equal quantities. The staining mixture is a nuclear stain and did not prove satisfactory for staining myelin sheaths in brain.—H. A. Davenport.

MONNE, LUDWIK. Polarisationsoptische Untersuchungen über den Golgi-Apparat und die Mitochondrien männlicher Geschlechtszellen einiger Pulmonaten-Arten. *Protoplasma*, 32, 184-92. 1939.

By using as intense an illumination as possible (panphoto microscope of Leitz with a strong low voltage lamp) the author was able to observe with crossed nicols that the Golgi-apparatus especially of *Helix pomatia* is doubly refractive. This condition was intensified by replacing the isotonic (0.7-0.8%) salt solution as medium for a more concentrated one (1-2%). Better results were obtained by staining with rhodamin B and 6G, but the cells stained with chrysoidin were best. With a sufficient concentration of chrysoidin (concentration not given) the Golgi-apparatus shows up beautifully white against a black background. The same occurs with the stained mitochondria and lipoid layers and fibrillae which, however, do not light up if unstained. In spermatocytes and spermatids the golgi elements are straight or curved rodlets surrounding the centrosome. Between crossed nicols the rodlets successively light up by rotating the nicols.—Robert Chambers.

NIKLITSCHEK, A. Das Tuschpräparat. *Mikrokosmos*, 33, 27. 1939.

The India ink method, discovered by Ehrenberg (1838), is fully reviewed. The ink may be rubbed up with dist. water in a depression of a white porcelain plate using a clear glass rod. Addition of ink should stop when the color of the ink creeping up on the margin changes from brown to jet black; trituration, however, should continue for at least 15 min. longer. The following applications are discussed:

Dry method: For blood parasites, a drop of blood is mixed with a drop of the ink on one side of the slide. The smear is then spread as in ordinary blood work, and allowed to dry. The preparation may be examined under oil immersion lens.

Wet method: For protozoa an equal amount of culture fluid and India ink are mixed. The criterion of a successful preparation rests in the proper distribution of the ink present. Immediately after placing the coverglass on the preparation, ring the mount with paraffin or Venetian turpentine. Cultures may thus be kept alive for a long time and studied at intervals.

When preservatives are added to the ink, they have a toxic effect upon the protozoa. This property may be advantageous in certain cases. When it is desired merely to slow the activity of protozoa, the addition of 0.5-4.0 g. of gum arabic in 10 ml. dist. water (that used in preparing the ink) will produce the desired results. Capsules of microorganisms may be clearly demonstrated.—J. M. Thuringer.

PASTERNACK, JOSEPH G. A reliable one-hour method for the preparation of paraffin sections of tissues. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 8-13. 1940.

The following fixation and embedding methods are recommended: Fix tissue in boiling 10% formalin, 1 min. Rinse a few seconds in tap water. Cut 1-2 mm. Blot dry. Preheat the reagents. A desirable vapor pressure is obtained by screwing the lids on the containers as soon as the tissue is inserted. Place in abs. alcohol at 56° C., 10 min.; acetone U.S.P. at 56° C., 10 min.; xylene 2 changes at 56° C., 5 min. each. Put in paraffin at 60-62° C. in a vacuum container and hold at this temp. for 10 min. Embed in paraffin and put in ice water while the paraffin is still melted. Cut 5-8 μ . Float on water at 45-50° C. Affix to slide with Mayer's fixative. Wipe back and edges of the slide. Put on hot plate at

50° C. for 3 min. Gently pass over a micro-burner to melt the paraffin. Remove paraffin with xylene from a dropping bottle. Before xylene has entirely evaporated cover the section with 0.2% celloidin in abs. alcohol and ether (1:1). Drain off excess fluid. Blow briskly to hasten evaporation. Immerse in water a few seconds to harden celloidin.

The staining methods recommended are: (1) Weigert's iron hematoxylin, 3 min.; rinse quickly in water; Van Gieson's, 1 min.; 95% alcohol, 10 sec.; acetone U.S.P., 10 sec.; xylene, 10 sec.; mount in balsam. (2) Harris' hematoxylin, 1 min.; 1% ammonia water until blue; rinse quickly in tap water; eosin Y, 15 sec.; rinse in 95% alcohol; acetone U.S.P., 10 sec.; xylene, 10 sec.; mount in balsam.—George H. Chapman.

SAIER, ELEANOR, and COBURN, WILLIAM. A modification of the dioxane dehydration method. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 18, 71. 1938.

The authors have developed a modification of the dioxan dehydration method, using the following procedure: Fix in Zenker's fluid or 10% formalin; wash and trim; leave in 95% alcohol from the time received (noon or before) until afternoon of the same day; place in dioxan over night; paraffin, 2-3 hr.; 2nd paraffin, 2-3 hr.; embed, section, and stain. Blocks received by noon are ready to be sectioned by afternoon of the following day.

The dioxan may be recovered by decanting from the CaCl₂ and filtering with suction into a flask containing 10 g. anhyd. CuSO₄ per liter of filtrate. After standing several days, the filtrate is distilled fractionally, and the dioxan is recovered between 101° and 103° C. More than 50% of the dioxan may be recovered in this way.

In embedding and sectioning of surgical specimens, the above method is recommended for the following reasons: It has cut the routine embedding time from six to three days; it is slightly less expensive if the dioxan is recovered; it is easier to handle; in embedding blood clots and organs of small animals, it prevents the gritty hardness that results from the use of xylene.—Jean E. Conn.

TURNER, OSCAR A. A manual of neurohistologic technique. (Third installment in series). *J. Lab. & Clin. Med.*, 24, 991-1003. 1939.

Procedures for the following staining methods are given:

(1) For connective tissue: Mallory's acid-fuschin-anilin-blue-orange-G stain; Haythorn's modification of Mallory's stain; Perdrau silver impregnation; Wilder method for reticulum; Karfield-Achúcarro tannin-silver method.

(2) For pituitary and pineal staining: Spark's method; Bailey's ethyl-violet-orange-G stain; Mallory's acid-fuschin-anilin-blue method; safranin-acid-violet stain; Hortega's stain for pineal parenchyma.

(3) For spirochetes: Dieterle method; Nieto's method; Jahnel's gold-silver method.

In addition to staining procedures, a discussion of the advantages and disadvantages of each method is included.—Jean E. Conn.

TURNER, OSCAR A. A manual of neurohistologic technique. (Fourth, and last, installment of series). *J. Lab. & Clin. Med.*, 24, 1096-1108. 1939.

Details are given for a miscellaneous group of staining methods including calcium and iron in tissues, supravital staining for rapid tumor diagnosis, staining of Negri bodies, gross staining of the brain for anatomy or pathology, and frozen sections of complete organs. Standard formulae are given for a number of common fixing and staining solutions. The use of nitrocellulose as an embedding agent is discussed and tables of dye solubilities and specific gravity of alcohol-water mixtures are included.—John T. Myers.

DYES AND THEIR BIOLOGICAL USES

BANK, O., und BUNGENBERG DE JONG, H. G. Untersuchungen über Metachromasie. *Protoplasma*, 32, 489-516. 1939.

For a study of coacervates, the authors investigated the nature of metachromatism, using toluidine blue and indigo carmine Ia as examples of basic and acid dyes respectively. Neutral violet, neutral red, janus green, nile blue,

brilliant cresyl blue, methylene blue, methylene green, and trypaflavin were also used as basic dyes (dye cation). As acid dyes (dye anion) orange G and erythrosin were employed.

The above-mentioned basic dyes were found to lose their metachromasy in dil. solutions but to become metachromatic in dil. solution when gum arabic or other negatively charged colloids are added. Inorganic anions may also induce metachromatism, especially ammonium paramolybdate and sodium phosphotungstate which give large complexes of polyvalent anions.

The disappearance of metachromatism is brought about by adding electrolytes with effective cations, the polyvalent cations being more efficient than the monovalent. An exception is the H-ion.

For acid dyes the metachromasy is enhanced by substances containing organic cations, e.g. clupein, chinin, strychnin, etc.

The authors conclude that metachromasy is a function of concentration of the dye in which aggregation of the dye molecules is facilitated when the concentration is high. The aggregation is due to van der Waal forces since the metachromasy disappears by the addition of alcohol, the effect increasing with the length of the carbon chain of the alcohol.—*Robert Chambers*.

BROH-KAHN, R. H. The bacteriostatic action of sulfanilamide under anaerobic conditions. *Science*, 90, 543-4. 1939.

The question of whether sulfanilamide acts as a bacteriostatic agent only in the presence of oxygen has long been a controversial point. The work reported in this paper substantiates claims that sulfanilamide may be bacteriostatic under anaerobic conditions. The evidence obtained from experiments with *Escherichia coli* may be summarized as follows: *E. coli* grows well aerobically in broth but poorly under anaerobic conditions; it is presumed to obtain energy partly from an aerobic, and partly from an anaerobic mechanism. If sulfanilamide inhibits only the aerobic mechanism, the degree of growth in the presence of oxygen and the inhibitor ought to approximate that obtained anaerobically—and such is the case. Sulfanilamide does not affect the action of glucose on growth obtained in the presence or absence of oxygen. It inhibits anaerobic growth in lactate-nitrate synthetic medium but does not affect aerobic cultures, thus displaying an activity similar to that of the cyanides. The conclusion is that bacteriostasis from sulfanilamide cannot be attributed to any non-specific mechanism, and that interpretation of its activity should be based upon performance under a variety of conditions in different media.—*J. A. de Tomasi*.

GERSHBERG, H., and FORBES, J. C. Precipitation of insulin with rhodamine-B. *Proc. Soc. Exp. Biol. & Med.*, 42, 95-6. 1939.

The authors have previously discovered that rhodamine B would precipitate pepsin, and later discoveries by others have shown that safranin precipitates insulin. Insulin is now found to be readily precipitated by rhodamine B at pH 7.2. The complex is dissolved in acid and the dye can be recovered with isoamyl alcohol, leaving insulin in the aq. layer. The insulin content of the washed ppt. formed on the addition of dye to a buffered solution of insulin has been demonstrated physiologically.—*M. S. Marshall*.

HOBBS, BETTY CONSTANCE. The part played by bacteria in the reduction of methylene blue in milk. *J. Dairy Research*, 10, 35-58. 1939.

The organism reducing methylene blue most rapidly in milk belonged to the coliform group, followed in decreasing order by *Streptococcus lactis*, and some fecal streptococci, *Staphylococcus aureus*, *Staph. albus*, *Staph. citreus*, some micrococci, group C hemolytic streptococci and some strains of *Streptococcus agalactiae*, and aerobic spore-formers. The methylene blue reduction in milk containing actively growing bacteria is considered by the author almost entirely the result of the metabolic reactions proceeding at the cell surface of the bacteria themselves. The methylene blue reduction test as used under practical conditions is considered a good index of the extent of bacterial metabolism in the milk.—*H. Macy*.

MODELL, W. Chlorazol fast pink BKS as an anti-coagulant. *Science*, 89, 349-50. 1939.

Clotting is a disturbing factor in kymograph experiments, where blood pressure of animals is to be recorded. Among many azo dyes, Chlorazol fast pink BKS

(C. I. 358) proves to be a particularly efficient anticoagulant, inexpensive as well as effective. Inasmuch as the crude dye (trade names: fastusol pink BBA; calcomine fast pink 2BL) is toxic it must be freed of salts and impurities. Purification is based upon precipitation from aq. solution by alcohol by the following technic: dissolve dye 1:15 in water, filter, add equal vol. of 95% alcohol; filter; wash ppt. with 70% alcohol; dry over steam and grind to a coarse powder. The yield is about 20% of the crude dye. A 5% solution should be used. A single intravenous dose of 100 mg. per Kg. prevents clotting for many hours. A method to avoid intravenous injection is proposed which calls for 0.5 cc. portions of the dye solution introduced at 30 min. intervals into the pressure system of the recording manometer just above the junction of the cannula and the rubber tubing.—*J. A. de Tomasi.*

NICHOLS, AGNES A. Bacteriological studies of spray-dried milk powder.
J. Dairy Research, 10, 202-30. 1939.

The methylene blue reduction tests at 37° C. and 55° C. were made on 405 samples of spray-dried milk. The weighted mean reduction time at 37° C. was 8.1 hr., varying from 3-14 hr. or more. At 55° C. the shortest time was 4.5 hr., and over 90% of samples required 12 hr. or more for decolorization.—*H. Macy.*

SMITH, W. S. The excretion of phenol red in the dogfish, *Squalus acanthias*.
J. Cellular and Comp. Physiol., 14, 857. 1939.

The kidney tubules of the dogfish freely excrete phenol red; the maximum excretion being reached at a phenol red concentration of about 2 mg. per 100 ml. of plasma. Hippuran depresses the rate of phenol red excretion by the tubules, while creatinine has no marked effect.—*L. Farber.*

ANIMAL MICROTECHNIC

ARMITAGE, F. L. A modified peroxidase stain for blood and bone marrow films. *J. Path. & Bact.*, 49, 579-80. 1939.

A method useful in distinguishing various cells in films of leukemic blood and sternal puncture blood marrow is as follows: Fix in 96% alcohol containing 10% formol, prepared immediately before use. Flood films with benzidine-H₂O₂ mixture (a filtered solution of 0.75 g. benzidine in 500 cc. 40% ethyl alcohol, with 7 cc. 3% H₂O₂ added and mixed immediately by shaking). Allow freshly-made films to stain 2 min., older ones longer. Control staining by washing with 40% alcohol until granular cells show definite yellow granules. Wash with 40% alcohol, abs. alcohol, dry in incubator. Counterstain with Leishman or dilute Giemsa. Wash with dist. water, blot dry.—*S. H. Hutner.*

HADJIOLOFF, A. Coloration des lipides au moyen de solutions hydrotropes de Sudan et d'autres lipocolorants. *Bull. d'Histo. Appl.*, 15, 37-42. 1938.

Seeking to avoid the formation of precipitates of fat stains, and to avoid any loss of fat due to the action of the fat stain solvent, the author has turned to the use of aq. solutions of the stains of the Sudan series (Sudan II, Sudan III, Sudan IV, Sudan red, Sudan black, etc.). Stains were obtained from Holborn, I. G. F. and Ciba.

These aq. solutions are termed "hydrotropic solutions" after Neuberg, who showed that certain substances previously dissolved in dist. water have the property of bringing other insoluble substances into solution. Hydrotropic agents for the fat stains include soaps, caffeine citrate, caffeine benzoate, saponin, sodium trioleate, trichloracetic acid and sulfosalicylic acid. To make such a hydrotropic solution the fat stain is added to a conc. solution of the hydrotropic substance and kept in an oven at 56° C. for several days. After filtering once or twice, a perfectly clear solution results.

Frozen sections of formalin fixed material were employed. They were stained between 20 min. and 24 hr., carefully rinsed and mounted in glycerin.

An important fact from the histochemical point of view is that most of the fat stains change their color from red to deep blue in hydrotropic solution, in proportion to the increase in concentration of the hydrotropic agent used. For example, using sulfosalicylic acid as the agent, the color varies, with increasing concentration of the solution, from red, thru violet, to deep blue. Such a blue hydrotropic solution, however, upon entering fat or a fat solvent again becomes red. Thus

the author considers any red granules in a cell following treatment with a blue solution of one of these Sudan dyes as being truly of a fatty nature.—*M. Noble Bates.*

HEILBORN, O. A new method of making permanent smears with special reference to salivary gland chromosomes of *Drosophila*. *Lantbrukshög-skolans Ann.* (Sweden) 4, 89-97. 1937.

There are several drawbacks to the usual aceto-carmine smear technic for chromosomes: the tissue elements adhere poorly to the slide and are often lost when making permanent mounts; changes in the technic are practically impossible; the staining solution promptly weakens under the coverslip; the process of sealing and breaking the mount to make it permanent is not satisfactory. The following smear technic for *Drosophila* material dissociates fixation from staining: Dissect the larvae in a drop of Ringer's solution and prepare the salivary gland with two needles, of which one at least should be bent at a right angle. Transfer the glands to a drop of 50% acetic acid, fix 4-8 min., and remove to a dry slide. Cover with a coverslip wet with glycerin; hold it fast, and smear with the aid of a needle or a small roller. Suspend the inverted slide in 96% alcohol in a Petri dish until the coverglass drops off (in about 3 min.). Rinse in water. Stain overnight (15-25 hr.) in aceto-carmine. Wash in water, dehydrate, clear, and mount as usual. The method is fairly rapid, yields smears that are permanent from the beginning, and can also be applied to pollen mother cells.—*J. A. de Tomasi.*

LENDRUM, A. C. A new trichromic staining method. *J. Path. & Bact.*, 49, 590-2. 1939.

The author modifies Masson's stain by replacing saffron, a dye of uncertain composition, with a solution of tartrazine in ethylene glycol monomethyl ether (cellosolve). The technic is as follows: Stain in haemalum; then in one of the following solutions: (A) eosin Y 0.1 g., erythrosin 0.1 g., phloxin 0.1 g., dissolved in 25 cc. of ethyl alcohol, and mixed with 25 cc. of 1% gallic acid containing 1% sodium salicylate; or (B) eosin Y 0.25 g., phloxin 0.25 g., dissolved in 35 cc. of 95% ethyl alcohol, with 15 cc. commercial formalin added. Stain in "A" 1-2 hr.; or in "B" 3-4 hr. Rinse with alcohol and add tartrazine cellosolve solution (a sat. solution tartrazine N.S., Imp. Chem. Ind., in commercial cellosolve). This replaces eosin with tartrazine. When the balance between red and blue is satisfactory, transfer to abs. alcohol, clear, and mount.

The best fixative is formol-sublimate (saturated aq. $HgCl_2$, 9 parts; commercial formalin 1 part, or 10% formalin followed by mordanting a few days in saturated $HgCl_2$). If the tissue is otherwise fixed or fails to show proper differentiation, leave unstained sections over night in formol-sublimate saturated with picric acid. After brief rinsing with water, iodine and $Na_2S_2O_3$, stain as above. For some tissues the tartrazine may be replaced advantageously by a solution of fast green F.C.F. in cellosolve.—*S. H. Hutner.*

LILLIE, R. D. Some experiments with the Masson trichrome modification of Mallory's connective tissue stain. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 18, 75. 1938.

The author reports on experiments undertaken in an attempt to shorten Masson's modification of Mallory's connective tissue stain without impairing the results. He reaches the following conclusions: (1) Formalin material may be satisfactorily stained by Masson's modification if sections are first mordanted with Bouin's fluid, picric acid or $HgCl_2$. (2) Mordanting for 10 min. at 58° C. in picric acid or $HgCl_2$ is as satisfactory as over-night mordanting in Bouin's fluid. (3) Various blue and green dyes of the sulphonated triphenylmethane group may be used satisfactorily as collagen stains.—*Jean E. Conn.*

FLINN, MACKAY. A rapid staining method for opsonocytophagocytic indices. *J. Lab. & Clin. Med.*, 25, 316. 1939.

The stain is made by adding 0.15 g. methyl green and 0.5 g. pyronin to 15 cc. 95% alcohol, preferably in a mortar. The stain is completely dissolved by slowly adding 85 cc. of 3% phenol. This mixture should stand a week. Air-dried smears are flooded with the stain for 4 min., washed in tap water and air-

dried. The cytoplasm of the leucocytes is faint pink, the nucleus light red and the organisms deep red. As the cytoplasmic granules do not stain, confusion is avoided.—*John T. Myers.*

PRYCE, D. M. Staining reticulocytes for demonstration purposes. *J. Path. & Bact.*, 49, 594-7. 1939.

Permanent and relatively undistorted preparations of reticulocytes may be made as follows: Prepare a small quantity of sat. solution of cresyl blue in rigidly anhyd. abs. alcohol, and use while fresh. Dip grease-free slides in this solution and air-dry, or spread with another slide. Spread blood on this cresyl-blue slide, and cover blood film immediately with another slide (the "spreader" may be used) which has a strip of gummed paper attached to the under side at each end, thus preventing contact between the two slides but delaying evaporation. After 5 min. at room temp., remove covering slide, dry film, and apply Leishman's stain undiluted for 5 sec. to 5 min. Dilute and stain another 10 min. Wash off stain with dist. water until the slide just changes from blue to red. The water should be slightly acid. Leishman staining removes excess cresyl blue.

An alternative method is to treat the slide with cresyl blue for only 1 cm. at one end, place the drop of blood at this end and spread slowly so as to allow the blood to be colored blue. Hold the spreader in this position for 2 min., then complete spreading of the film, and dry. Reticulocytes stain in all parts of the film and excess stain is avoided.—*S. H. Hutner.*

PRYTHERCH, HERBERT F. The life cycle and morphology of Nematopsis ostrearium, sp. nov., a gregarine parasite of the mud crab and oyster. *J. Morphol. & Physiol.*, 66, 39-66. 1940.

The author describes a rapid method of preparing smears and whole mounts of host tissues containing gregarine parasites. The procedure is as follows: Kill small seed oysters, previously kept out of water for several hours, by freezing them in the shell. Then remove pieces of expanded meats or mantle, fix in BS (a modification of Bouin's solution containing urea and chromic acid), wash and dehydrate. Stain by flooding with pinacyanol dissolved in abs. alcohol (conc. not given), destain in lower alcohols, and stop at desired depth by immersion in water. Place slides in staining dishes containing sodium silicate for $\frac{1}{2}$ hr. or more. Then apply cover glass and seal edges with marine glue.

The usual methods involving fixation in BS, and staining in Heidenhain's iron-hematoxylin, Mayer's haemalum, or Delafield's hematoxylin are also described.—*Elbert C. Cole.*

SHEEHAN, H. L. The staining of leucocyte granules by Sudan black B. *J. Path. & Bact.*, 49, 580-1. 1939.

To bring out the granules of leucocytes, the following technic is recommended: Dry blood film at least 15 min. before fixation, and fix $\frac{1}{2}$ min. in methyl alcohol. Stain 1 hr. in sat. Sudan black B in 70% alcohol. Rinse in water, wash 1 min. with 70% alcohol to remove deposit of dye. Counterstain $\frac{1}{2}$ min. with sat. solution "alcoholic" eosin in 70% alcohol. Rinse in water, stain 3 min. in sat. aq. methylene blue, rinse, blot dry. The granules in the leucocytes are deep black and are not decolorized by 70% alcohol or mounting in Canada balsam.—*S. H. Hutner.*

WALKER, THOMAS F., and SWEENEY, PATRICIA A. A method of counting blood platelets. *J. Lab. & Clin. Med.*, 25, 103-4. 1939.

The following technic is proposed: Draw a 1.1% solution of sodium oxalate to the mark 1 in a white blood cell pipette and expel it. Draw the blood to mark 0.5, and the diluting fluid (1.1% sodium oxalate) to mark 11, and mix. Place a heavy rubber band around the pipette to cover both ends and centrifuge just enough to make the red cells settle (usually 80 sec. at 1600 R.P.M.), or let stand upright for about 2 hr. Gently expel the red cells by blowing. Place a drop of the clear fluid in the counting chamber. After 10 min., count all the platelets in 80 small squares, and add 3 zeros to get the number of platelets per cu. mm.—*John T. Myers.*

PLANT MICROMECHANIC

KASSANIS, BASILIOS. Intranuclear inclusions in virus infected plants. *Ann. Appl. Biol.*, 26, 705-9. 1939.

Two kinds of intracellular inclusions in solanaceous plants infected with severe etch virus are described. One occurs in the cytoplasm and is similar to the X-bodies found in many other plant virus diseases. The other occurs only in the nuclei. These intranuclear inclusions appear to be crystalline, and have the form of thin rectangular plates.

The most suitable fixative for the intranuclear inclusions was found to be formol-saline (20 parts formalin, 80 parts 0.9% NaCl solution). Fixatives containing acetic acid, picric acid or alcohol do not give good results as they coagulate the proteins. Also, the first dissolves the crystals. Flemming's solution without acetic acid gives good results. The crystals stain readily with acid dyes such as acid fuchsin and eosin. The simplest method for differentiating the intranuclear crystals is to mount an epidermal strip in an aq. solution of eosin. The best method for staining fixed preparations is that of Kull, using acid fuchsin, toluidine blue and aurantia, which was used by Paillot (1926) in working with the polyhedral disease of silkworms. The crystals and the nucleoli stain red, and the chromatin blue. With Heidenhain's hematoxylin, the crystals stain black, and when placed in solutions of iron alum, retain their color longer than any other of the cell contents.—F. M. Clark.

MÜHLDORF, ANTON. Über die Bildung und Auflösung der Wände bei der Tetradeteilung der Pollenmutterzellen von *Althea rosea*. *Ber. deut. botan. Ges.*, 57, 299-312. 1939.

In a study of the cell wall structure of *Althea rosea*, the best preparations were made from fresh anthers crushed in weak neutral red which stained the protoplasm lightly. Anthers fixed in formol or Nawaschin's formol-chromic-acid mixture and crushed with weak neutral red gave fair slides. Paraffin sections stained and mounted in balsam were very poor.—Merritt N. Pope.

RÄMSCH, HEINZ. Entwicklungsformen und Degeneration im Xanthoria-Apothecium. *Arch. Mikrob.*, 10, 279-301. 1939.

For a microscopic study of *Xanthoria* apothecia the following technic is proposed: Place apothecia in water until swollen, and fix under a vacuum 3-20 hr. in a mixture of 0.06% osmic acid, 0.06% glacial acetic acid and 0.2% chromic acid. Wash 2-3 days in running water and run up thru alcohols from 30% to absolute. Transfer to cedar oil for at least 8 hr. in vacuum, then to celloidin of 5, 10, 30 and 50% (24 hr. for each step). Add celloidin shavings to the 50% stage until syrup is thick. Leave object in this for several days, and embed in paper tray. Harden by hanging paper tray in 85% alcohol for 24 hr. and store in 70% alcohol. Before cutting, place in 60% alcohol where a long stay improves the cutting. While 3 μ sections are easily obtainable, 6 μ to 9 μ is recommended. Stain with iron alum hematoxylin and counterstain for 5 min. with rubin S. After the iron alum, differentiate with weak grades of alcohol until the chromatin appears blue-green and the protoplasm a bright red. Good results were obtained also from Herman's safranin and gentian-violet method. Erythrosin as a single stain shows the cell membrane.—Merritt N. Pope.

WERGIN, W. Über den Aufbau pflanzlicher Zellwände. V. Mitteilung: Untersuchungen über die Baueinheiten mit Hilfe der Quellungsanalyse. *Protoplasma*, 32, 116-39. 1939.

After a short review of the literature (33 citations) on the microscopic structure of cellulose walls of plant cells and on methods hitherto used for causing a separation by swelling of the cellulose lamellae, the author presents his own method as follows: Cotton fibers, stretched into straight filaments by weighting their ends, are given a preliminary coating of celloidin followed by imbedding in paraffin. The sections are made as nearly as possible along the long axis of the fibers. The sections are mounted on slides without any adhesive material, the paraffin and celloidin are removed by means of alcohol-ether while the sectioned fiber is held in place by the needles of a micromanipulator. The exposed sectioned fibers are further treated by mounting in water between cover-slip and slide. The solution, run under the coverslip to induce the swelling of the cellulose lamellae, consists of 0.58 g. Ca(OH)₂ in 100 cc. of 25% ammonia containing a variable amount

of NaOH (about 0.4%), the concentration to be varied for regulating the swelling process.—*Robert Chambers.*

MICROORGANISMS

DAVIS, J. G., McCLEMONT, J., and ROGERS, H. J. *Studies in Mastitis. I. The routine diagnosis of mastitis.* *J. Dairy Research*, 10, 60-73. 1939.

Crystal violet (1:500,000) was used in Edward's medium to inhibit organisms other than that of mastitis.—*H. Macy.*

GUITTONNEAU, M. G., and BÉJAMBES, M. *Chromo-résistance et enrobage phosphocalcique des microbes chauffes dans le lait.* *Le Lait*, 19, 225-34. 1939.

Streptococcus thermophilus cells were obtained from a 16-hr. milk culture by centrifuging, resuspended in low-count raw or heated milk at the rate of 10 cells per ml., and the suspension heated for one hour at 100° C. or 20 min. at 120° F. When smears from milk which had not been heated at high temperatures before adding the cells were stained with a sat. aq. solution of methylene blue, the cells remained unstained. They were stained when the milk had been heated at high temperatures before inoculating and then reheated. The chromo-resistance was eliminated by treating the cells with dil. acetic acid or CO₂. The chromo-resistance of *S. thermophilus* acquired thru heating in fresh milk was attributed to adsorption, by the membrane of the bacterial cell, of calcium phosphates of milk liberated from the colloidal medium by heat treatment.—*H. Macy.*

HSU, C. L., and TUNG, T. *Bactericidal action of X-rays in the presence of dyes.* *Proc. Soc. Exp. Biol. & Med.*, 42, 828-30. 1939.

It is suggested that the mechanism of ordinary photosensitization and that of the lethal effect of X-rays on bacteria suspended in dye solutions may be the same, viz., oxidation. Experimental evidence was based on tests with mercurochrome, eosin, methylene blue, crystal violet and safranin O, in which were suspended various Gram-negative or Gram-positive bacteria. Exposure to X-rays was direct, with adequate controls for temperature and for possible lethal effects of dyes or X-rays as such. Bactericidal action under radiation was, in some instances, as much as 1000 times that of the dye alone. Eosin with a non-lethal quantity of H₂O₂ showed remarkable activity when irradiated either with visible rays or X-rays. Visible light apparently may be stronger or weaker than X-rays, depending on the dye and the organisms.—*M. S. Marshall.*

KLIMMER, M., and WEISKE, GERTRUD. *Zur Züchtung der Galtstreptokokken aus Milch. IV. Selektive Nährböden.* *Milchw. Forsch.*, 19, 15-22. 1937.

The authors studied, among other compounds, trypaflavin, alizarin, acid yellow, Bismarck brown, tropaeolin OO, pyronin, martius yellow, naphthol yellow, aurantia, nile blue sulfate, brilliant green, fast green, fuchsin, iodine green, crystal violet, malachite green, methyl violet 5 B, Victoria blue 4 R, in agar containing sucrose, serum, alkaline albuminate, and brom cresol purple (Klimmer, Haupt and Roots) to determine inhibition of mastitis streptococci and other organisms in milk.—*H. Macy.*

KNISELY, M. J. *A simple and time saving procedure for the identification of Treponema pallidum.* *J. Lab. & Clin. Med.*, 24, 1909. 1939.

The procedure recommended employs a colloidal aq. solution of silver sold under the name of "Collargolum", which is diluted 1:20. The technic is as follows: Place a drop of the suspected material on a slide and mix with an equal volume of the reagent. Streak out as for a blood smear. Examine microscopic ally when dry. The unstained *Treponema pallidum* stands out against a background of dark yellow to light brown.—*John T. Myers.*

LAWSON, GEORGE McL. *Modified technique for staining capsules of Hemophilus pertussis.* *J. Lab. & Clin. Med.*, 25, 435-8. 1940.

The writer recommends the following technic: Allow smears to air-dry; cover them with 5% aq. phosphomolybdic acid for 30 sec.; wash in water, and then in methyl alcohol. Cover with 10-20 drops of stain (Wright stain 2 parts and glycerol 1 part) for 2 min.; then add 20-30 drops of dist. water and let stand

10-20 min.; rinse, dry, and examine. Much better capsules are obtained if the culture is grown on Bordet-Gengou medium containing 5-8% of mucin.—*John T. Myers.*

McCLEMENT, J., and DAVIS, J. G. Studies in Mastitis. IV. Mastitis in relation to the methylene blue reduction test. *J. Dairy Research*, 10, 88-93. 1939.

The methylene blue reduction test was not capable of detecting mastitis. Reduction time was more closely related to the cell content of the infected milk.—*H. Macy.*

MILAKNIS, ANTANAS. Beitrag zur elektiven Züchtung des *Streptococcus agalactiae* und der *Brucella Bang*. *Milchw. Forsch.*, 19, 392-6. 1938.

Crystal violet in a 1:3,000,000 concentration in broth was used to determine inhibition of growth of *Streptococcus agalactiae*, a *Brucella* strain and several saprophytes. It was effective against Gram-negative bacteria and all Gram-positive rods or filamentous types, but four different *Streptococcus* strains and two *Micrococcus* strains were resistant and overgrew the mastitis strain.—*H. Macy.*

NOVEL, E. Une technique facile et rapide de mise en évidence des cils bactériens. *Ann. Inst. Pasteur*, 63, 802-11. 1939.

A reliable technic is offered for staining bacterial flagella: Clean new slides in bichromate-sulfuric-acid; rinse; soak in xylene 24 hr.; then store in strong alcohol. To use, remove slide with forceps, burn off alcohol, and place on a plate at 50° C. Prepare the bacterial emulsion by removing a particle of the growth on an agar slant (avoiding water of condensation) and immerse the loop for 20-30 sec. in 2-3 cc. of water in a grease-free watch-glass. Place 3-5 drops of the emulsion separately on a slide. When drops are dry, remove slide without further fixation. Prepare fresh mordant as follows: Tannin 20%, 50 cc.; cold sat. FeSO₄, 40 cc.; sat. alc. solution basic fuchsin or gentian violet, 7 cc. Filter the mordant from a small paper and funnel onto the slide so as to cover all the dried areas. Allow to stand 1-1½ min. Rinse in dist. water and apply freshly prepared Fontana-Tribondeau Ag-impregnation solution (AgNO₃, 1 g.; water, 20 cc.; with 10% NH₄OH added to the solution drop by drop until the ppt. formed redissolves, but without going beyond point of opalescence). When preparation is light chocolate-colored (usually between 50-80 sec.) rinse in dist. water and dry on warm plate. The flagella stand out strongly, and only 3 min. are required for the whole procedure.—*S. H. Hutner.*

RITTER, CASSANDRA. Studies of the toxicity of basic fuchsin for certain bacteria. *Amer. J. Pub. Health*, 30, 59-65. 1940.

Six dyes which are included in the general term basic fuchsin were tested for bacteriostatic titer against 10 strains of bacteria (coliform types, a streptococcus, and aerobic spore formers) which might be found in water. Two of the dyes were commercial basic fuchsins certified by the Biological Stain Commission: DF-4, pararosanilin acetate or a mixture of acetate and chloride, dye content 91%; and CF-15, rosanilin chloride, dye content 91%. The other four were specially prepared chloride salts of the 4 lower basic members of the magenta series, designated as follows: pararosanilin chloride (Magenta O); rosanilin chloride (Magenta I); Magenta II; and new fuchsin (Magenta III). The dyes were tested in such a way as to determine the bacteriostatic titer under the same conditions for each member of the dye series; this gave a relative titer, comparing each dye with the others, the results not being absolute for any specific dye and organism combination. The results for all organisms bore a relative titer corresponding to the number of methyl groups in the dye radical, the titer increasing with the larger number of methyl groups. The commercial samples showed bacteriostatic titers only slightly higher than those of the pure salts of which they were largely composed, suggesting the presence in these samples of small amounts of the higher homologs. Results on another series of certified commercial basic fuchsin, composed of both acetate and chloride salts, suggested that the anion has small part in determining the bacteriostatic titer. Basic fuchsin is used in fuchsin lactose broth for water analysis in a concentration of 0.0015% or 1:66,000. This concentration of pararosanilin or of rosanilin would eliminate unwanted spore-forming bacteria but not inhibit the coliform group, according

to the experiments here reported. Either rosanilin (Magenta I) or pararosanilin (Magenta O) may be used in fuschin lactose broth for bacteriostatic action, the pararosanilins being preferable. Magenta II and III are not suitable. All samples of basic fuchsin submitted for certification up to this time have been found satisfactory in accordance with the results of these experiments. Tests of new batches of dye may be made by determining the bacteriostatic titer, in comparison with the titer of the standard dye, for one coliform group organism and one aerobic spore former.—*M. W. Jennison.*

STOVALL, W. D., and BLACK, C. E. The influence of pH on the eosin methylene blue method for demonstrating Negri bodies. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 8. 1940.

A solution of 1% ethyl eosin in 95% alcohol stained Negri bodies pale red at a reaction of pH 6.0 or more alkaline. The intensity increased at more acid reactions, reaching a maximum at pH 3.0. Loeffler's methylene blue, used as a counterstain, was most satisfactory at pH 5.3; it removed eosin at a pH-value of 6.0 or higher. The following technic is recommended:

Stain 2 min. in 1% ethyl eosin in 95% alcohol with 5.5% N/10 HCl added. Rinse in dist. water. Stain 2 min. in the following solution: sat. alc. methylene blue (medicinal), 15 cc.; dist. water, ad. 60 cc.; sodium acetate, 0.4 g.; glacial acetic acid, 0.08 cc. Rinse with dist. water. Rinse in dil. acetic acid (dist. water, 60 cc. with 18 drops of glacial acetic acid) until the color of the section changes from deep blue to brownish red. Rinse in dist. water. Run thru graded alcohols, keeping the section in abs. alcohol until the color no longer washes out, controlling the differentiation by observation under the microscope. Run into xylene, and mount in balsam. This technic gives a higher proportion of positive results. Detailed directions are given for selecting the brain tissue, preparing it, fixing, dehydrating, infiltrating in paraffin, embedding, cutting, sectioning and staining.—*George H. Chapman.*

SULLIVAN, N. P., and SEARS, H. J. A simple technique for concentrating tubercle bacilli in sputum. *J. Lab. & Clin. Med.*, 24, 1093-5. 1939.

Approximately 0.5 g. papain is mixed with 50 cc. sputum, and incubated at 37° C. till liquefied (usually 10-15 min.). The material is centrifuged and smears are prepared from the sediment in the usual way. The sediment may be used for animal inoculation or cultures without further treatment.—*John T. Myers.*

HISTOCHEMISTRY

BRODA, B. Über die Verwendbarkeit von Chinalizarin, Titangelb und Azoblaau zum mikro- und histochemischen Magnesiumnachweis in Pflanzengeweben. *Mikroskopos*, 32, 184. 1939.

A histochemical technic for demonstrating magnesium is proposed, which calls for the following reagents: (1) Quinalizarin, 1 part, triturated with 5 parts sodium acetate crystals; employed as fresh (not over 24 hr.-old) 0.5% solution in 5% aq. NaOH. (2) Titan yellow; 0.2% aq. solution. (3) Azo blue; 0.1% aq. solution. (4) NaOH; 10% aq. solution.

Technic: On each mounted paraffin section, place 1-2 drops of stain reagent 1, 2, or 3, adding 1-2 drops of 10% NaOH solution in the case of reagents 2, and 3. Put on a cover glass, and examine under a comparison microscope, with pure magnesium lake, to facilitate the detection of small quantities of magnesium. Magnesium is usually found in the globoids of aleuron granules. The typical staining appears in a few minutes. Oils interfere with the reaction and should be removed previously with alcohol-ether mixture. Quinalizarin, after several hours, produces a distinct blue stain, titan yellow stains brick red to rose, and azo blue stains violet.

The glandular cells of medicinal plants which contain magnesium give only a slightly stronger reaction than the ordinary basal cells which likewise contain magnesium. Sections of fresh stems of sugar cane are first brought into 95% alcohol before being treated with the reagents. Large irregular blue stars may be observed in the interstitial spaces. *Panicum virgatum* (millet) serves as test material for determination of magnesium. (Leaves, stems, roots, and shoots may be used.) Quinalizarin in strong ammoniacal solution may be used as a reagent for the detection of calcium oxalate (e.g., in stems of rhubarb, *Rheum rhabonticum*).—*J. M. Thuringer.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the January number of this Journal.

STAINS CERTIFIED DEC. 1, 1939 TO FEB. 29, 1940*

| Name of dye | Certification No. of batch | Dye Content | Objects of tests made by Commission† | Date approved |
|----------------|----------------------------|-------------|--------------------------------------------------------------------------|---------------|
| Giemsia stain | LGe-1 | | As blood stain | Dec. 4, 1939 |
| Thionin | NT-6 | 85% | As histological stain and for frozen tissue | Dec. 7, 1939 |
| Basic fuchsin | LF-5 | 94% | For general staining, the Feulgen reaction, and in bacteriological media | Dec. 26, 1939 |
| Cresyl violet | NW-8 | 86% | For use in histology | Jan. 16, 1940 |
| Wright stain | LWr-9 | | As blood stain | Jan. 16, 1940 |
| Toluidine blue | NU-3 | 59% | For general histological staining | Jan. 8, 1940 |
| Methyl green | NG-14 | 78% | In histology and as constituent of Pappenheim stain | Jan. 19, 1940 |
| Resazurin | NRz-1 | | For testing reduction in milk | Jan. 23, 1940 |
| Wright stain | NWr-14 | | As blood stain | Feb. 7, 1940 |

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAIN TECHNOLOGY

VOLUME 15

JULY, 1940

NUMBER 3

AN OSMIC IMPREGNATION METHOD FOR MITOCHONDRIA IN PLANT CELLS

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Osmium tetroxide, in technics like Kolatchev's (1927-8) and its various modifications, has been used for years in animal cytology for the preservation of various protoplasmic constituents, such as the Golgi apparatus. Bowen (1927-8), using the Kolatchev technic on plant tissues, described a new category of cytoplasmic inclusions

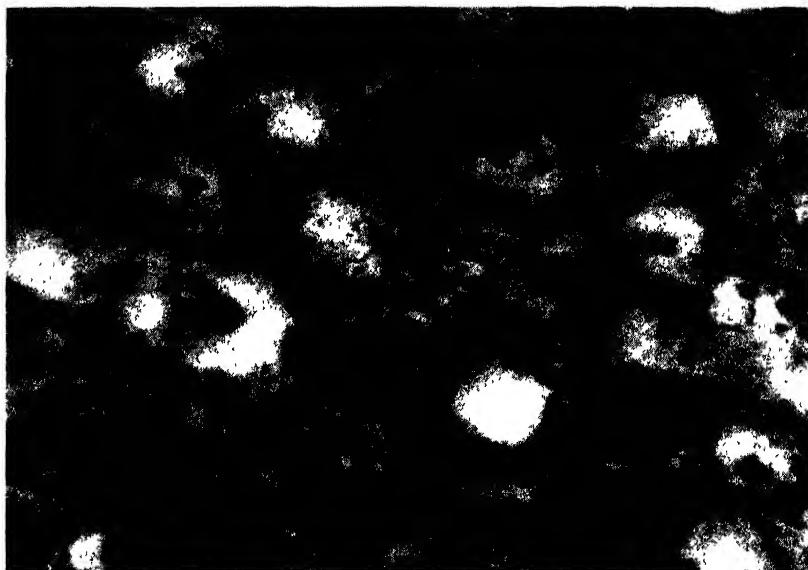


Fig. 1. Photomicrograph of root-tip cells of *Pisum*, $\times 1425$. The preparation is unstained.

which he called the "osmiophilic platelets." The author (1940), in duplicating some of Bowen's work, used this technic and observed the bodies described by Bowen which he considered to be deformed chondriomes caused largely by the initial fixation. A simple modification of the technic, however, in which Zirkle's fluid was substituted for Champy's, resulted in preparations in which the chondriome and

¹Journal article No. 362, n.s.

proplastids were beautifully preserved and selectively blackened by the osmium tetroxide, showing clearly the transition stages between the two categories. No staining is necessary after bleaching, the chondriome and proplastids appearing jet black against the light gray background of cytoplasm and nuclei (Fig. 1). The nuclei, cytoplasm and cell walls can be stained with Altmann's mixture of acid fuchsin and anilin oil (Lee, 1937, p. 305) followed by a counterstain of gold orange in clove oil. The result is a brilliant triple stain, with the nuclei red, cytoplasm and cell walls orange, and the chondriome black.

A summary of the technic is as follows:

1. Cut root-tips or other material directly into Zirkle's² fixative and fix for 48 hours.
2. Wash 8 hours or over night in running tap water.
3. Treat with 2% OsO₄ for 4-6 days—change solution on alternate days. (It was found impossible to standardize this step of the technic, because root-tips in the same bottle will exhibit different rates of reduction of the OsO₄.)
4. Wash 8 hours or over night in tap or distilled water.
5. Dehydrate by the usual alcoholic series substituting benzene for xylene in clearing, or by the shorter *N*-butyl-alcohol-ethyl-alcohol series of Lang (1937).
6. Embed in 52° paraffin and section at 5 μ .
7. Bleach as follows: 1% KMnO₄, 5 minutes. Rinse in distilled water, then place in 3% oxalic acid for 2-3 minutes. Wash in running tap water for 15 minutes.
8. Dehydrate thru graded alcohols, clear in xylol and mount in balsam. Or, if the preparation is to be stained, pass from water into acid fuchsin (pipette acid fuchsin on slide, heat until steaming, let stand for 5 minutes, repeating the procedure once more, and when cool, wash in water), and ascend thru the alcohols, counter-staining in 1% gold orange in clove oil after absolute alcohol. After the counterstain, wash and clear in xylol and mount in balsam.

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²Zirkle's fixative: 8% K₂Cr₂O₇..... 1.25 g.
3% (NH₄)₂Cr₂O₇..... 1.25 g.
3% CuSO₄..... 1.00 g.
Distilled water..... 100.00 g.

THE DETERMINATION OF APPARENT ISOELECTRIC POINTS OF CELL STRUCTURES BY STAINING AT CONTROLLED REACTIONS¹

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ABSTRACT.—The staining reactions at controlled pH-values of various dyes with the nucleus and cytoplasm of *Trichonympha collaris* under different conditions were investigated. When staining intensity was plotted against pH, it was found that with each dye a different curve was obtained. "Isoelectric points" obtained by superposition of acid and basic dye curves varied for the same material with the dyes employed. It was found that, with the same dye, the curves of staining intensity plotted against pH varied with the buffer system utilized. Moreover, the intensity of staining at any pH was found to vary directly with the concentration of dye and inversely with the concentration of buffer. Various factors modifying staining intensity were studied. In the staining of a protein in buffered solution, it was shown that staining intensity (the index of the concentration of the dye-protein compound) at a given pH-value is dependent upon the interaction of the dye-protein, buffer-protein and dye-buffer systems, and that as the dye or buffer or their concentrations were varied, the resultant "isoelectric points" which were obtained also varied. In view of these facts and of the present lack of knowledge of dyes and dye-protein combinations it would be impossible to determine a true isoelectric point by staining at controlled pH-values without further extensive work on the subject. It follows that no true isoelectric points have hitherto been obtained for nucleus, cytoplasm or other tissue elements by staining at controlled pH.

INTRODUCTION

Since Loeb (1922) showed that it was possible to approximate the isoelectric point of gelatin by staining it with certain acid and basic dyes in a series of buffered solutions, a number of workers have applied this method to determine the "isoelectric points" of the nucleus and cytoplasm of various tissues.

The theory underlying this method of determining isoelectric

¹From a dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Zoology in the Graduate Division of the University of California.

points is as follows: If the amount of dissociation of a protein or other ampholyte is plotted against pH, a curve will be obtained having two maxima and one minimum. The pH at which the minimum occurs is defined as the isoelectric point of the protein. On the more acid side of the isoelectric point (i.e., at lower pH-values), the protein is dissociated as a positively charged ion. On the other side of the isoelectric point, it is dissociated as a negatively charged ion. Near the isoelectric point, however, there will be a certain small percentage of negative ions on the acid side, and a similar small percentage of positive ions on the basic side, since the dissociation curves of the acid and basic groups of the protein are sigmoid in shape, and the isoelectric point is the point at which the two cross.

Acid dyes dissociate to form negatively charged dye ions. Basic dyes dissociate into positively charged dye ions. Therefore the acid dyes will combine with the positively charged protein ions at pH-values below the isoelectric point, and basic dyes will combine with the negatively charged protein ions at pH-values above the isoelectric point. Assuming the acid dye to be completely dissociated, or nearly so, if a series of dye solutions is set up at different pH-values and samples of a protein are stained in them, the intensity of staining will depend on the amount of ionization of the protein. Hence if intensity of staining is plotted against pH, a sigmoid curve should result, which indicates the amount of dissociation into positive ions of the protein. Similarly, with a basic dye a curve is obtained which indicates the amount of dissociation of the protein into negative ions. The isoelectric point should be the point at which these two curves cross. It will be noted that the above discussion is based on the stoichiometric theory of staining. This is done merely for the sake of convenience, as the adsorptive theory would be equally applicable. For a more detailed discussion than is given here, the paper of Craig and Wilson (1937) may be consulted.

Previous workers, staining tissues as outlined above, have found that with acid dyes the intensity of staining decreases as the pH increases, that with basic dyes the intensity increases with increasing pH, and that the curves obtained are roughly sigmoid. With this confirmation of the theory, they have proceeded to determine isoelectric points for the nucleus and cytoplasm of a wide variety of tissues. None of them, however, has attempted a systematic investigation of the effects of various factors on the isoelectric points obtained. In view of the number of papers on the subject which have been published, and of the significance of the results if valid, it was determined to subject the method itself to a critical evaluation. Fac-

tors which, it was considered, might be important were (1) the dye used, (2) the concentration of dye used, (3) the buffer salts used, and (4) the concentration of buffer used. Each of these was varied independently of the other three, and the results of such treatment noted.

HISTORICAL

Pischinger (1926) made the first attempt to determine the isoelectric points of tissue elements. Practically all later workers used his method or a modification of it. He stained gelatin, egg albumin, and alcohol-fixed thymus and cartilage with toluidine blue (a basic dye) and cyanol extra (an acid dye) at a series of pH-values, and plotted the logarithm of the amount of stain against pH. The amount of dye taken up was determined by extracting after staining, and using a colorimeter.

He also stained sections on slides of alcohol-fixed tissues. The slides were stained ten minutes and washed briefly with the proper buffers. The toluidine blue preparations were placed in 4% ammonium molybdate to prevent extraction of stain in alcohol. Most of the water was removed with filter paper, and the slides passed thru xylene-alcohol and xylene to balsam. Stain intensity was estimated by microscopic inspection. Pischinger gives a table of isoelectric points and ranges for the nucleus, cytoplasm and tissue elements of various tissues. These differ for different tissues.

In one case information is given on the effect of buffer concentration on the staining. With toluidine blue, Pischinger found that the neurofibrils stained more intensely and in a more acid region in M/20 buffers than in M/200 buffers. The stain intensity of the former at pH 5.2 was about equal to that of the latter at pH 6.0.

Methyl violet 6B, safranin, Congo red, and picric acid were also used. Pischinger states that the results with safranin and picric acid were in general the same as those for toluidine blue and cyanol extra respectively. With methyl violet, the isoelectric point was shifted toward the acid side. With Congo red, there was a slight decrease in staining on the alkaline side, but even at pH 7.6 (the highest used) the tissues contained a considerable amount of dye. At no pH-value were the nuclei stained, but the dye was precipitated below pH 3.3, so Pischinger could go no lower.

In spite of these contradictory results, Pischinger believed that he had obtained true isoelectric points with toluidine blue and cyanol extra. In a later paper (1927) on the isoelectric points of muscle constituents, he changed his dyes to methylene blue and crystal ponceau.

Other investigators were Pulcher (1927), who worked with smears of frog blood, guinea pig blood, egg albumen, fresh fibrin and "stromi di emazie"; Mommsen (1927), Schwartz-Karsten (1927) and Ochs (1928), who worked with air-dried human blood streaks; Tolstoouhov (1927, 1928), working with blood smears and tissue sections; and Pfeiffer (1929), who worked with plant tissues.

Zeiger (1930a, b) continued Pischinger's work by investigating the effect of the fixing fluid on the isoelectric points of a number of tissues. He used M/200 methylene blue and crystal ponceau as his basic and acid dyes. Each fixative gave a different isoelectric point for the same tissue, but with different tissues the shift in isoelectric point as compared with that of absolute alcohol was not invariably in the same direction. Altho, as this paper will show, these so-called "isoelectric points" obtained by Zeiger are not the actual isoelectric points of the tissues, nevertheless his paper is of interest. The relative shifts in isoelectric range are possibly valid.

Others who used similar methods to obtain isoelectric points of various tissues were Yasuzumi (1933a, 1933b, 1934), Nishimura (1934), Yasuzumi and Matsumoto (1936), Sturm (1935), Achard (1935), Fautrez (1936) and Ikeda (1935, 1936).

A lengthier historical account is given by Levine (1937).

METHOD

The material selected for the investigation was the hypermastigote flagellate, *Trichonympha collaris*. This protozoon is to be found in great abundance in the intestine of the damp-wood termite *Zootermopsis angusticollis*, which is common in the San Francisco Bay area. Thousands of organisms are present in one termite. Coverslip smears were made by removing the hindgut, breaking its wall, and distributing the exuding material as evenly as possible on the cover-slip with a pair of dissecting needles. In order to cleanse their intestines of wood particles, whose color would obscure the staining reactions, the termites were previously fed on filter paper for at least a week before use.

Before any drying could take place the smears were dropped into a fixative. Five different fixatives were used, Susa's, Schaudinn's, Bouin's, absolute alcohol, and liquid air. The first three fixatives were used at 60° C., the absolute alcohol at room temperature. Fixation lasted 30 minutes except with liquid air, in which an immersion of 30 seconds was sufficient. Material fixed in liquid air could not be stained at pH-values above 5.0, because the low acidity allowed the protozoa to come off the coverslips. Liquid air fixation

was also unsatisfactory with acid dyes. The protozoa stained readily, but the stain came out quickly in the subsequent washing process. Therefore no isoelectric points could be calculated for this fixative. With this exception, all fixatives were used in all stains.

After fixation the coverslips were washed in running tap water for an hour to remove the fixatives, and then transferred to the staining solutions. After staining for 36 hours, at which time equilibrium was very nearly approached, the smears were washed thoroly in distilled water. Then the coverslips were passed thru four changes of tertiary butyl alcohol and two changes of neutral xylene, and mounted in neutral Canada balsam. The tertiary butyl alcohol prevented the loss of stain which would have taken place in ethyl alcohol. (Levine, 1939).

All staining solutions were prepared in the same way. Dye and buffer solutions of twice the concentration required were made up, and equal quantities of each were mixed to obtain the final staining solutions. The actual pH-values of the solutions were determined by means of a glass electrode. The addition of dye changed the pH-values of the buffers, the amount of change depending on the dye and on its concentration. In order to obtain buffer or dye solutions of different concentrations, the concentrations of the stock solutions were varied, or the buffered solutions were diluted as required.

The following series were run:

1. The effect of different dyes on the "isoelectric point" obtained.

Fixatives:—Susa's, Schaudinn's, Bouin's, absolute alcohol, liquid air.

Buffer:—McIlvaine's citric-acid-secondary-sodium-phosphate buffers diluted ten times (referred to hereinafter as 1/10 McIlvaine's buffers), at approximate pH values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.

Dyes:—Basic dyes—0.0001 *M*² toluidine blue O (National Aniline Co., Cert. No. NU-2, dye content 60%), 0.0001 *M* methylene blue U. S. P. medicinal (National Aniline Co., Cert. No. NA-48, dye content 89%), 0.0001 *M* Nile blue sulfate (National Aniline Co., Cert. No. NNb-1, dye content not given), and 0.0001 *M* crystal violet (A. H. Thomas Co., no certification number or dye content given).

Acid dyes—S/20³ponceau 2R (Coleman and Bell Co., no certification number or dye content given), and 0.0001 *M* orange G (National Aniline Co., Cert. No. NO-3, dye content 85%).

²The concentrations of dye solutions were made on the basis of the dye contents of the dry dyes, except in the cases of Nile blue sulfate, crystal violet and ponceau 2R, for which dye content was not known. In these cases it was arbitrarily assumed to be 80%.

³When a 0.002 *M* stock solution of ponceau 2R was made up, it was found that the dye did not all dissolve even after several days. This solution was then filtered, and the filtrate considered a saturated solution. The symbol S/20 thus indicates a 1/20 saturated solution.

2. The effect of concentration of dye on the isoelectric point obtained.

Fixatives:—As above.

Buffer:—As above.

Dyes:—The same six dyes were used as above, in concentrations of 0.0001 *M*, 0.00025 *M*, 0.0005 *M*, and 0.001 *M*. In the case of ponceau 2R the concentrations were S/20, S/8, S/4, and S/2.

3. The effect of different buffers on the isoelectric point obtained.

Fixatives:—As above.

Buffers:—All buffers were diluted to one-tenth their standard strength.

- (1) 1/10 McIlvaine's buffers (citric acid and secondary sodium phosphate) at pH-values approximately 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0.
- (2) 1/10 Sörensen's buffers (sodium citrate and HCl) at pH-values approximately 1.0, 2.0, 3.0, 4.0 and 5.0.
- (3) 1/10 Sörensen's buffers (primary potassium phosphate and secondary sodium phosphate) at pH-values approximately 5.0, 6.0, 7.0 and 8.0.
- (4) 1/10 Clark and Lubs' buffers (potassium acid phthalate and HCl) at pH-values approximately 3.0 and 4.0.
- (5) 1/10 Clark and Lubs' buffers (potassium acid phthalate and NaOH) at pH-values approximately 5.0, 6.0 and 7.0.

Dyes:—(1) 0.0001 *M* toluidine blue O.

(2) S/20 ponceau 2R.

4. The effect of different concentrations of buffer on the isoelectric point obtained.

Fixatives: As above.

Buffers:—The same buffers were used as in the preceding series. McIlvaine's and Sörensen's citrate-HCl buffers were used in standard, 1/10 standard, and 1/100 standard concentrations, the other buffers in standard and 1/10 standard concentrations.

Dyes:—(1) 0.0001 *M* toluidine blue O.

(2) S/20 ponceau 2R.

To determine intensity of staining, color charts were prepared as follows: A series of eight small squares was ruled off on white drawing paper. The first square was painted evenly with a 1% solution of one of the dyes, the second square with a 0.5% solution, the third with a 0.25% solution, and so on down to the last square, which was faintly colored by the 1/128% dye solution. Thus a geometric progression in intensity was obtained, each step being double the intensity of the preceding one. These charts had approximately the same shade of color as the slides, except in the case of crystal violet, in which the stained organism contained more red than did the color chart. The difference was not sufficient to interfere with color comparison, however. With the aid of these charts, the intensity of staining of the *Trichonympha* nucleus and cytoplasm under the various experimental conditions was recorded. The methylene blue chart was used on

organisms stained in Nile blue sulfate and on the nuclei stained in toluidine blue O, since these dyes gave its color and not those of their own charts. While this method involves the use of the eye in comparing intensities, it was found that after some practice a high degree of accuracy was obtained as judged by the results of repeated determinations made on the same material at different times.

RESULTS

The experimental values obtained were plotted on graph paper with stain intensity on the ordinate, and pH on the abscissa. From these graphs, "isoelectric points" were obtained and tabulated. In

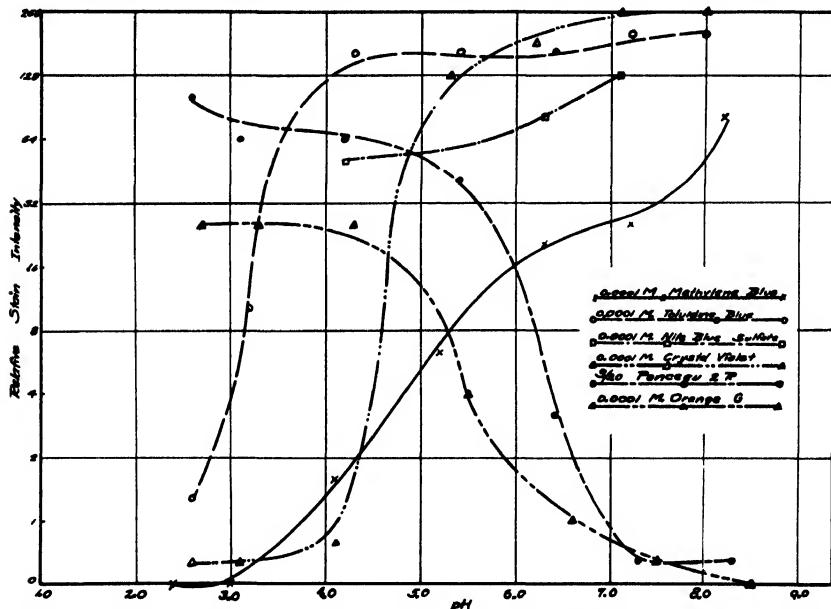


Fig. 1. The effect of different dyes on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1-10.

all, 136 graphs were constructed and used, but it is obviously impracticable to present more than a few representative ones in this paper. All the other graphs are to be found in the writer's doctorate thesis (Levine, 1937).

SERIES 1. THE EFFECT OF DIFFERENT DYES ON THE "ISOELECTRIC POINT" OBTAINED.

In Fig. 1 stain intensity is plotted against pH for the six dyes used with *Trichonympha collaris* nucleus after Susa's fixation.

Theoretically, if the curves determine isoelectric point, those of the four basic dyes should all be the same curve or approximately the

same, and those of the two acid dyes should be the same. The pH-value at which these two ideal curves crossed would be the isoelectric point of the cell element. This is obviously far from the fact.

Table 1 gives the "isoelectric points" obtained from the intersection of the acid and basic curves for the nucleus of *Trichonympha collaris*. With any given fixative, the isoelectric point should be the same regardless of the dyes used in obtaining it. Therefore all the figures in each vertical column of each table should be the same. Far from this, Table 1 gives values for the nucleus fixed in Susa's ranging from 3.3 to 6.0, with Schaudinn's from less than 2.6 to 4.9, with Bouin's from 3.8 to 5.6, and with absolute alcohol from much less than 2.6 to 4.8. Similar results were obtained for the cytoplasm.

TABLE 1. "ISOELECTRIC POINTS" OF TRICHONYMPHA COLLARIS NUCLEUS

| Dye Combination* | Fixative | | | |
|------------------|----------|-------------|---------|--------------|
| | Susa's | Schaudinn's | Bouin's | 100% Alcohol |
| P-TB | 3.6 | 3.5 | 4.7 | 3.3 |
| OG-TB | 3.3 | 3.0 | 4.2 | 3.0 |
| P-CV | 4.9 | 3.0 | 4.7 | <<2.6* |
| OG-CV | 4.7 | 2.9 | 4.5 | <<2.6 |
| P-NBS | 4.9 | 3.1 | 4.7 | <2.6 |
| OG-NBS | <4.2 | <2.6 | 3.8 | <2.6 |
| P-MB | 6.0 | 4.9 | 5.6 | 4.8 |
| OG-MB | 5.3 | 3.9 | 5.3 | 4.3 |

*The symbols used in the table are:

P = Ponceau 2R

CV = Crystal violet

OG = Orange G

NBS = Nile blue sulfate

TB = Toluidine blue O

MB = Methylene blue

<< = "much less than"

Even if the results with only one acid dye are used, the range becomes only a little narrower. For instance with ponceau 2R as the acid dye, the isoelectric points of the nucleus range with Susa's fixation from 3.6 to 6.0, with Schaudinn's from 3.0 to 4.9, with Bouin's from 4.7 to 5.6, with absolute alcohol from much less than 2.6 to 4.8. Similar results are obtained with the cytoplasm, or with orange G substituted for ponceau. With this assortment of "isoelectric points," it would seem difficult to pick the correct one, if any is correct.

Certain facts appear from the data. First, orange G gives a consistently lower "isoelectric point" with any of the basic dyes than does ponceau 2R. Second, methylene blue gives a consistently higher "isoelectric point" with either of the acid dyes than does any other basic dye. The other three basic dyes do not arrange them-

selves in any consistent consecutive order. Third, when the fixation is the same, the "isoelectric points" of the cytoplasm are consistently higher than those of the nucleus. This is in accord with the results of Pischinger (1926, 1927), Zeiger (1930 a, b), Craig, and Wilson (1937), etc.

SERIES 2. THE EFFECT OF CONCENTRATION OF DYE ON THE ISOELECTRIC POINT OBTAINED.

In Fig. 2 stain intensity is plotted against pH for different concentrations of the same dye. The results obtained on *Trichonympha collaris* nucleus fixed in Bouin's and stained with toluidine blue O are shown. The curves obtained with the other combinations of dyes and fixatives are essentially similar in their variability.

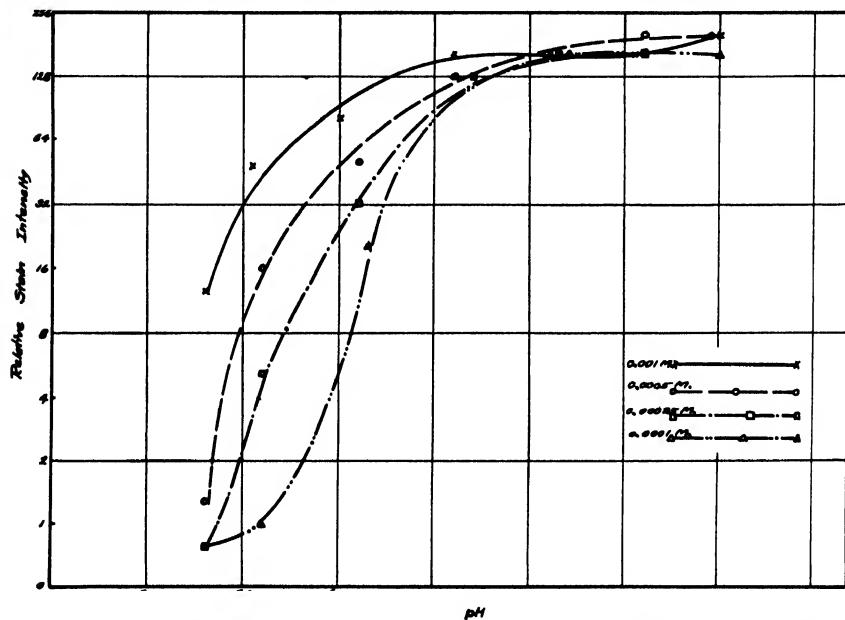


Fig. 2. The effect of dye concentration on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Bouin's. Dye—toluidine blue O. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1-10.

An analysis of these graphs shows that different concentrations of dye give different curves, which may be more or less widely separated. Usually, the higher the concentration of basic dye, the further to the acid side is the resultant curve, and the lower would be the "isoelectric point" obtained by its use. With acid dyes, the higher the concentration of dye, the further to the basic side is the resultant curve, and the higher would be the "isoelectric point" obtained; this point is illustrated in Fig. 3. These two opposite tendencies cancel each other out in part, but there is still a great discrepancy in the

resultant isoelectric points. In Table 1 are given the "isoelectric points" obtained for Schaudinn-fixed *Trichonympha* nucleus by superposition of the acid and basic dye curves. The degree of variability of "isoelectric points" of nucleus and cytoplasm with the other fixatives was essentially similar.

It is apparent that as the concentration of acid dye decreases, the pH of the "isoelectric point" also tends to decrease, and as the concentration of basic dye decreases, the pH of the "isoelectric point" tends to increase. This general rule has many exceptions, of which a large number are due to the positions of the points of intersection of

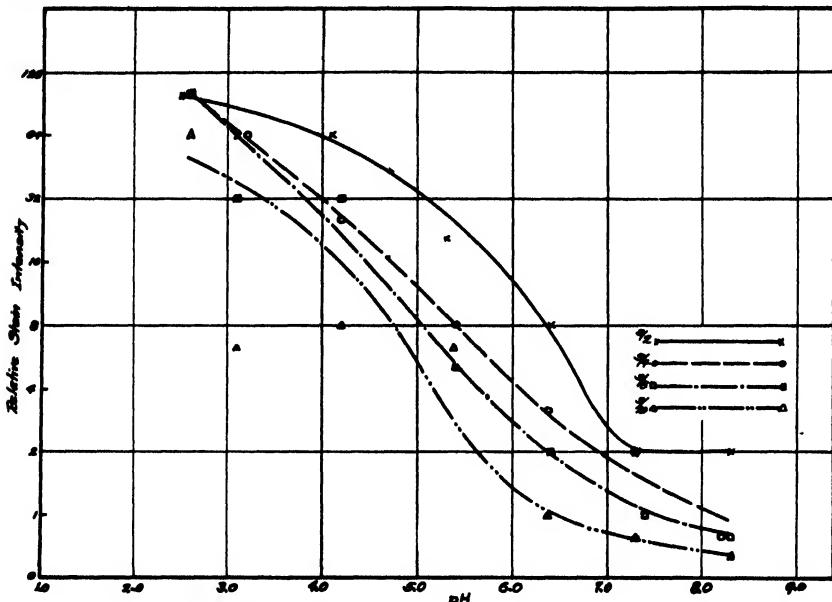


Fig. 3. The effect of dye concentration on the apparent isoelectric point of *Trichonympha collaris* cytoplasm. Fixative—Schaudinn's. Dye—ponceau 2R; dilution symbols (S/2, S/4, etc.), indicate degree of saturation. McIlvaine's buffers (citric acid and secondary sodium phosphate) diluted 1-10.

the acid and basic curves. If a true isoelectric point were being obtained, the two curves should intersect near their lower ends, for the isoelectric point represents a minimum amount of dissociation. But this is seldom the case. Most intersections occur near the upper extremities of one or both curves, some near the midpoint, and few below the midpoint. This is another point indicative that the true isoelectric point is not obtained.

SERIES 3. THE EFFECT OF DIFFERENT BUFFERS ON THE ISOELECTRIC POINT OBTAINED.

In figure 4 are shown the curves obtained with 0.0001 M toluidine blue O on Susa-fixed *Trichonympha collaris* nucleus. The results

TABLE 2. THE EFFECT OF DYE CONCENTRATION ON THE "ISOELECTRIC POINT" OF SCHAUDINN-FIXED TRICHOLOGYPHA COLLARIS NUCLEUS

| Acid Dye | Basic Dye | | | | | |
|------------|-----------|----------|-----------|----------|----------|-----------|
| | 0.001 M | 0.0005 M | 0.00025 M | 0.0001 M | 0.0005 M | 0.00025 M |
| Ponceau 2R | | | | | | |
| S/2* | 2.3 | 2.5 | 2.6 | 3.4 | 3.0 | 3.6 |
| S/4* | 2.6 | 2.7 | 2.7 | 3.3 | 3.2 | 3.5 |
| S/8* | 2.4 | 2.6 | 2.6 | 3.3 | 3.2 | 3.5 |
| S/20* | <2.4 | <2.5 | <2.6 | 3.1 | 2.8 | 2.9 |
| Orange G | | | | | | |
| 0.001 M. | <2.4 | <2.5 | <2.6 | 2.7 | <2.6 | 2.6 |
| 0.0005 M. | <2.4 | <2.5 | <2.6 | 2.8 | <2.6 | 2.6 |
| 0.00025 M. | <2.4 | <2.5 | <2.6 | 2.8 | <2.6 | 2.8 |
| 0.0001 M. | <2.4 | <2.5 | <2.6 | 2.7 | <2.6 | <2.6 |
| Acid Dye | Basic Dye | | | | | |
| | 0.001 M | 0.0005 M | 0.00025 M | 0.0001 M | 0.0005 M | 0.00025 M |
| Ponceau 2R | | | | | | |
| S/2* | 2.8 | 3.4 | 3.5 | 3.7 | 5.1 | 5.0 |
| S/4* | 2.6 | 3.3 | 3.4 | 3.6 | 4.9 | 4.8 |
| S/8* | 2.6 | 3.3 | 3.4 | 3.6 | 4.9 | 4.7 |
| S/20* | <2.4 | 3.0 | 3.1 | 3.4 | 4.8 | 4.5 |
| Orange G | | | | | | |
| 0.001 M. | <2.4 | <2.5 | <2.6 | <2.6 | 3.1 | 3.7 |
| 0.0005 M. | <2.4 | <2.5 | <2.6 | <2.6 | 3.4 | 3.9 |
| 0.00025 M. | <2.4 | <2.5 | <2.6 | <2.6 | 3.7 | 4.0 |
| 0.0001 M. | <2.4 | <2.5 | <2.6 | <2.6 | 2.6 | 2.9 |

*S/2 = half saturated solution, S/4 = quarter saturated solution, etc.

obtained with the other fixatives and with ponceau 2R are similar. It is seen that at a given pH the same stain intensity is not obtained with different buffers. Table 3 gives the "isoelectric points" of *Trichonympha* nucleus under different conditions of fixation and buffering. The isoelectric points were determined by superimposing the curves for ponceau 2R on those for toluidine blue O. With the nucleus, the range of "isoelectric points" obtained with the same fixative covers from 0.9 pH-units (Bouin's) to 1.7 pH-units (Susa's). A similar variation was observed with the cytoplasm. The buffers can be arranged in a regular series with regard to their effect on the

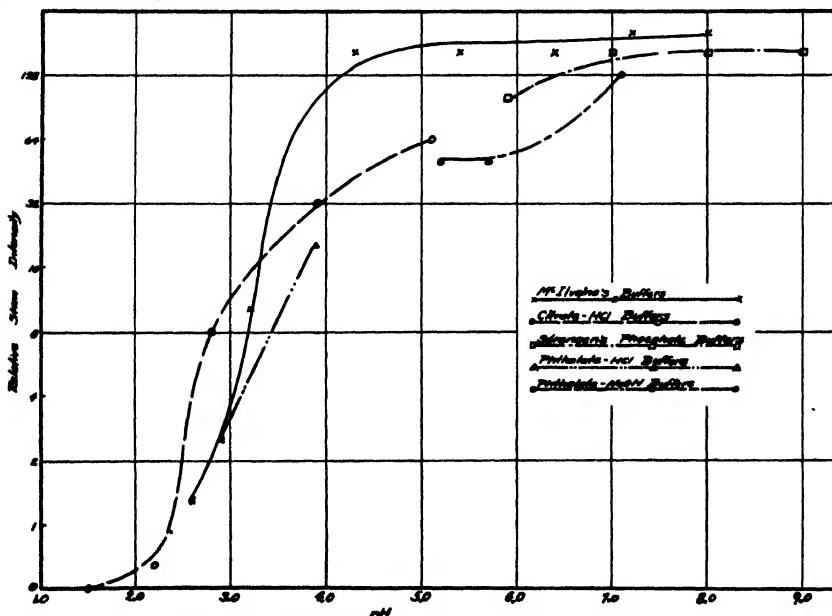


Fig. 4. The effect of different buffers on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. Dye—toluidine blue O. All buffers 1/10 standard concentration.

"isoelectric point." McIlvaine's buffers (citric acid and secondary sodium phosphate) give the lowest isoelectric points; Sörensen's buffers (sodium citrate and HCl) give higher isoelectric points; and Clark and Lubs' buffers (potassium acid phthalate and NaOH) give still higher ones. If the other two buffers had covered a wide enough pH range, they would undoubtedly have given still different "isoelectric points."

SERIES 4. THE EFFECT OF THE CONCENTRATION OF BUFFER ON THE ISOELECTRIC POINT OBTAINED.

Figure 5 shows the effect of the concentration of McIlvaine's buffer on the isoelectric point of *Trichonympha* nucleus, when

stained with toluidine blue O after fixation in Susa's. A similar variability was obtained with the other fixatives, with ponceau 2R and with the other buffers.

TABLE 3. THE EFFECT OF DIFFERENT BUFFERS ON THE "ISOELECTRIC POINT" OF *TRICHONYMPHA COLLARIS* NUCLEUS

| Fixative | Buffer | | |
|-----------------------|-------------|-------------|----------------|
| | McIlvaine's | Citrate-HCl | Phthalate-NaOH |
| Susa's | 9.7 | 4.6 | 5.4 |
| Schaudinn's | 3.6 | 3.9 | <5.2 |
| Bouin's | 4.7 | 4.5 | 5.6 |
| Alcohol | 3.4 | 3.7 | <5.2 |

The accuracy of the very dilute buffer curves (i.e., 1/100 the original concentration) is doubtful. It is very probable that in such dilute systems the reactions changed in the course of the staining process. If these are omitted from consideration, it is seen that as a general

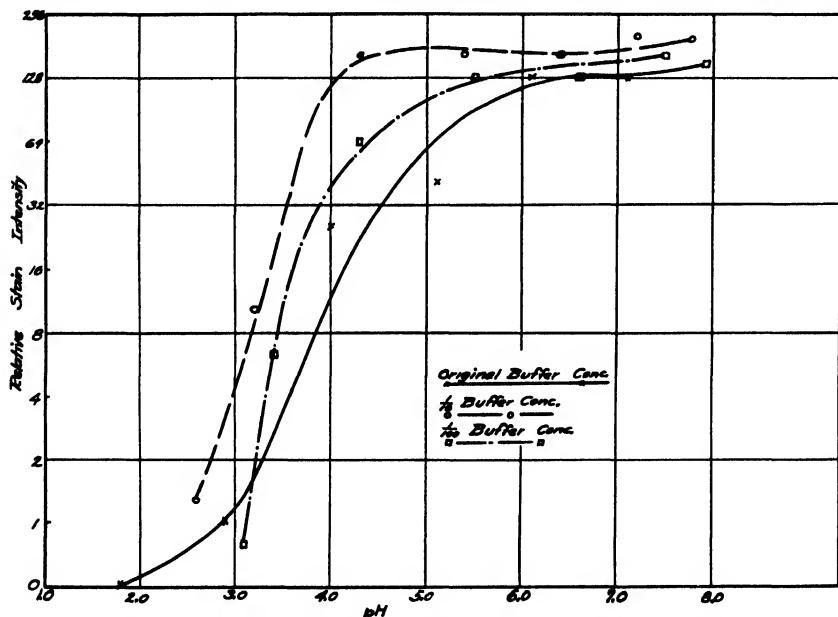


Fig. 5. The effect of buffer concentration on the apparent isoelectric point of *Trichonympha collaris* nucleus. Fixative—Susa's. Dye—toluidine blue O. McIlvaine's buffers (citric acid and secondary sodium phosphate).

rule the more concentrated the buffer, the less intense is the staining at any given pH-value.

Table 4 gives for *Trichonympha* nucleus the isoelectric points obtained from the graphs by superimposing the ponceau curves on the toluidine blue ones. The lowered staining intensity with increased buffer concentration would tend to produce a higher iso-

electric point with toluidine blue and a lower one with ponceau. These two opposite effects partially cancel each other out, but as a result the isoelectric points obtained have no constant relation to each other.

STAINING OF CASEIN

In order to check the experiments on *Trichonympha*, casein was stained at different pH-values with toluidine blue O and methylene blue. The casein, prepared by the method of Van Slyke and Baker and washed thirty days, was obtained from Dr. P. L. Kirk of the Department of Biochemistry, University of California. One-tenth-gram samples were placed in 10 cc. of 0.0003 M dye made up in 1/10 McIlvaine's buffers (citric acid and secondary sodium phosphate) at pH values approximately 2.2, 3, 4, 5, 6, 7, and 8. The relative amounts of stain taken out of solution by the protein at different reactions was noted after 24, 48, and 72 hours. There was no essential difference between the results at these times.

TABLE 4. EFFECT OF BUFFER CONCENTRATION ON THE "ISOELECTRIC POINT" OF *TRICHONYMPHA COLLARIS* NUCLEUS

| Buffer | Fixative | | | |
|-------------------------|----------|-------------|---------|---------|
| | Susa's | Schaudinn's | Bouin's | Alcohol |
| McIlvaine's | | | | |
| Standard Conc | 4.4 | 3.3 | 4.3 | 2.8 |
| 1/10 Conc | 3.7 | 3.4 | 4.3 | 3.3 |
| 1/100 Conc | 3.7 | 3.7 | 4.2 | 3.8 |
| Citrate-HCl | | | | |
| Standard Conc | >4.7 | 3.6 | >4.7 | 3.1 |
| 1/10 Conc | 4.7 | 3.9 | 4.6 | 3.7 |
| 1/100 Conc | 4.7 | 3.8 | 4.4 | 3.5 |
| Phthalate-NaOH | | | | |
| Standard Conc | 6.5 | 5.7 | 5.9 | <5.0 |
| 1/10 Conc | 5.4 | <5.2 | 5.6 | <5.2 |

At pH 6, some protein was dissolved; at pH 7 most of it was dissolved, and at pH 8 all the casein went into solution. These three pH-values were therefore valueless, since it was impossible to determine how much dye was combined with the protein, and how much was not. At any lower pH-value, however, it was found that relatively more methylene blue than toluidine blue was present in the supernatant fluid above the dye-protein precipitate. Therefore methylene-blue-casein is more completely ionized at these reactions than is toluidine-blue-casein. Hence the intensity of staining of casein is less with methylene blue than with toluidine blue. This result is in complete accord with the results obtained for *Trichonympha*.

DISCUSSION

In order to apply a staining method to the determination of iso-electric point, the cells must be killed and fixed. Unless the cell is dead, most dyes will not penetrate its outer membrane. Therefore in no case does the method establish the isolectric points of the living proteins, but only those of the proteins of the dead organism. Different fixatives, as Zeiger (1930b) has shown, give different isolectric points. It has been assumed that absolute alcohol fixation does not alter the isolectric points of tissue proteins. It is possible, however, that alcohol does in fact shift the isolectric point. Certainly it changes the nature of the proteins markedly.

Except in the case of blood cells, it has been necessary to section the tissues before they could be stained. Before sectioning, the fixed block of tissue must first be passed thru increasing grades of alcohol, xylene or toluene, and paraffin. Then, after mounting, the sections must again be passed thru xylene and alcohol to water before staining. This treatment undoubtedly has some effect on the proteins. The resultant effect is not that of the fixative alone. The effect of a post-fixation in alcohol, xylene, paraffin, etc., is added. The degree to which this may be a disturbing factor is not known. In the present investigation the necessity of sectioning was eliminated by the use of protozoan smears.

In all work reported on blood streaks, the streaks are air-dried before staining. Air-drying, as Seki (1934) has pointed out, makes the cell membranes more impermeable to staining solutions, and also has the more serious effect of markedly altering the "isolectric points" of the cell proteins. The experiments in which this took place are, however, of value with respect to the development of a practical, pH-controlled blood stain.

Most of the investigations of "isolectric point" have utilized concentrated dye solutions and a short staining period. A ten-minute immersion was the usual time, and equilibrium was not reached. This treatment introduces another unknown variable into the reaction, i.e., the rate of staining. Its elimination would be desirable. This can easily be accomplished by using dilute staining solutions, and allowing the reaction to proceed to equilibrium, as was done in the present work.

When the slides are run up thru alcohol before mounting, there is always a loss of stain in the alcohol. This is particularly serious in the case of methylene blue and toluidine blue, the basic dyes used by most investigators. Such a loss of stain would result in throwing the results far off. In order to prevent it as much as possible, slides

stained in these two dyes were fixed by these investigators in ammonium molybdate solution before being passed into alcohol. Altho this treatment is helpful, it nevertheless allows a great deal of dye to escape. For this reason, results previously reported with these two dyes should be viewed with some suspicion. Levine (1939), however, found that the substitution of tertiary butyl alcohol for ethyl alcohol in the dehydration process eliminated practically all loss of stain. This technic was used in the present work, and ammonium molybdate fixation was not found to be necessary.

To obtain the isoelectric point of a protein from its staining properties, the intensity of staining with an acid and a basic dye should be plotted against pH. Two opposite curves will be obtained, and the isoelectric point is theoretically the pH-value at which the curves cross. This is the basis on which Yasuzumi obtained his isoelectric points. Pischinger, Zeiger, etc., however, used only the curves for their basic dyes, and considered the isoelectric points to be the mid-points of these curves. This assumption is not valid.

Many investigators have not determined the pH-values of their staining solutions directly, but obtained the pH-values of their buffers colorimetrically and assumed that the addition of dye did not alter the reaction appreciably. It is a fact, however, that the addition of dye, particularly of as much dye as ordinarily used, may shift the reaction several tenths of a pH-unit. The glass electrode is the most satisfactory instrument for determining the pH of dye solutions, since hydrogen and quinhydrone electrodes are liable to be poisoned either by the dye itself or by unknown impurities present in it, and since the glass electrode has no tendency to drift. In the present investigation a glass electrode was used to determine the pH-values of the buffered staining solutions.

When a dye stains a protein, the intensity of staining is an index of the amount of dye-protein compound formed. According to the mass law, the amount of dye-protein compound is dependent on the concentrations of dye and protein ions in the reacting system. The concentration of dye ion is a function of the amount of dye present, its dissociation constant, and of pH. Similarly, the concentration of protein ion is a function of the amount of protein present, the isoelectric point of the protein, and the pH of the solution. If, then, dyes are chosen with sufficiently great dissociation constants to be completely ionized, or nearly so, over the pH-range in which they are used, at first glance it would appear possible to determine the isoelectric point of a protein by staining at varying pH-values.

Unfortunately, it is not such a simple matter. The solubility

product or dissociation constant of the dye-protein compound must be taken into consideration. This determines what proportion of the dye-protein compound itself is ionized at any pH-value. An example will show the importance of this factor. Take, for instance, two dye-protein compounds, AP and BP, in which the protein is the same but the dyes are different. At a given pH-value the original concentration of protein ions is the same for both systems. If the dyes selected are completely dissociated at this reaction (they can be selected so that they will be) and if the dye concentrations are the same, the amount of dye-protein compound formed will be the same in both cases. But if the dissociation constant of AP is greater than that of BP, at any pH-value below that at which both are completely dissociated and above that at which neither is dissociated, AP will be more dissociated than BP. In other words, more dye will be ionized from AP than from BP, and consequently the actual concentration of undissociated dye-protein compound will be less for AP than for BP. Hence, the resultant intensity of staining will be less with dye A than with dye B at the same pH. Hence when staining intensity is plotted against pH for the two dyes, different curves will result. Then if one plots curves for two basic dyes and one acid dye, and takes the isoelectric point as the pH at which the acid and basic dye curves cross, two different isoelectric points will be obtained. The more dyes used, the greater the number of "isoelectric points" which will be obtained. The use of different fixatives changes the nature of the proteins, introducing new isoelectric points and new dye-protein dissociation constants. This theoretical reasoning is confirmed by the experimental results obtained in the staining of casein with methylene blue and toluidine blue O (see p. 103), and also by the experimental results given in Table 1. The "isoelectric points" obtained with the different dye combinations range over more than two pH units. This fundamental deficiency of the method makes it impossible to obtain true isoelectric points by its use. No criterion is available to establish which particular combination of dyes gives a true isoelectric point, if any do.

Further confirmation is obtained from Fig. 1 in the paper of Rawlins and Schmidt (1929). Here are plotted titration curves of casein against methylene blue, safranin Y and indulin scarlet for pH-values from 6 to 12. In their experiments pH was established with HCl or NaOH, and not with buffers, and the dye-protein compounds were insoluble even at the high pH-values. The amount of combination was determined colorimetrically. From pH 7 to 12 the amount of dye-protein for the three dyes was approximately the

same. This is to be expected on the basis of the theory. At pH 6, however, the three curves separate. The casein binds 100×10^{-6} equivalents of methylene blue, 150×10^{-6} equivalents of indulin scarlet, and 175×10^{-6} equivalents of safranin Y per gram of protein. Had the curves been carried to a lower pH-value, the results would have been even more striking.

It follows from the mass law that the lower the dye concentration, the less dye-protein compound will be formed at any reaction. Hence, when different concentrations of dye are used, the curves for staining intensity plotted against pH should be different. With basic dyes, as concentration of dye decreases, the curve will be shifted toward a higher pH-value. With acid dyes, the curve will be shifted the other way. As a result, different "isoelectric points" will be obtained with different dye concentrations. A glance at Fig. 2 and Table 2 will show that even with the complex mixture of proteins in protoplasm the above prediction is confirmed.

The buffer system also has its effect on the staining curves. This is because the amount of dye-protein compound is dependent on the concentrations of ionized dye and protein which are free to combine at any reaction. The buffer salts are not inert, but react with both dye and protein. They bind a certain amount of both in the form of un-ionized dye-buffer and buffer-protein compounds. Hence the amount of free dye and protein ions is decreased by the buffer salts. The extent to which this takes place depends on the dissociation constants of the dye-buffer and buffer-protein compounds. The reasoning is similar to that employed for the dye-protein reaction. The smaller the dissociation constant of the dye-buffer compound, the more dye is inactivated by the buffer, and the less will be the quantity of dye-protein compound resulting. Similarly, the smaller the dissociation constant of the buffer-protein compound, the more protein will be inactivated by the buffer, and the less protein will be free to combine with the dye.

The dissociation constants of the dye-buffer and buffer-protein compounds depend in part on those of the buffer salts employed. Hence it would be expected that with different buffers, these dissociation constants would be different and that different amounts of dye and protein would be inactivated. Therefore when staining intensity is plotted against pH, different curves would result with different buffer systems. This is the case in the experiments described in the present paper. "Isoelectric points" almost two pH-units apart were obtained simply by varying the buffer system.

According to the mass law, if the concentration of buffer salts be

increased, more dye and protein should be taken up by the buffer system, and hence less dye-protein compound would be formed. Consequently, with basic dyes, the more concentrated the buffer, the further to the alkaline side the curve of staining intensity will be shifted. For acid dyes, with the more concentrated buffer, the curve will be shifted to the acid side. The experimental results of the present investigation show that this relation holds in the staining of protoplasm.

Temperature, too, plays a part in the reaction, since it changes the dissociation constants of the various compounds present. In short, if the temperature factor is ignored, the intensity of staining depends at a given pH-value upon the interaction of three systems, the dye-protein, dye-buffer, and buffer-protein. If any one factor changes, the intensity of staining also changes.

Any attempt to determine the isoelectric point of a protein by a staining method in the present state of our knowledge would be useless. Even if the dissociation constant of the dye were known, or if it were high enough to produce complete dissociation over the pH-range studied, the isoelectric point would be one of three unknown factors, the other two being the dissociation constants of the dye-protein and the buffer-protein compounds. These would depend in part on the dye and the buffer salts utilized, and therefore when these were varied the results would not be the same.

The above discussion applies to pure proteins. It should be emphasized that protoplasm is not a pure protein, but an extremely complex system of many kinds of proteins, carbohydrates, lipoids and salts. Its protein complex has no true isoelectric point, but rather a more or less broad isoelectric range which is the expression of the isoelectric points of the component proteins. Then, too, the lipoids, carbohydrates and salts are not inert. Reactions between certain dyes and certain of these other constituents would give misleading results. Undoubtedly such reactions occur. It is known that alizarin red S has a strong affinity for calcium. Craig has found (oral communication) that malachite green is precipitated by the phosphate ion. Both calcium and phosphate are always found in protoplasm. Any dye used to determine isoelectric point may, for all we know, have a similar reaction with some specific non-protein ion present in the protoplasm. In such a case it would be expected that different dyes would give different staining results. While the results obtained by Pischinger, Zeiger, the writer and others indicate that cytoplasm and nucleus have different isoelectric ranges and are composed of proteins whose isoelectric points are fairly close to each

other, little more can be said. Since it is at present impossible to find the true isoelectric point of a single protein by the staining method, it would be useless to try to use it to determine actual isoelectric ranges of protoplasmic elements.

SUMMARY

1. The staining reactions at controlled pH-values of various dyes with the nucleus and cytoplasm of *Trichonympha collaris* under different conditions have been investigated. When stain intensity was plotted against pH it was found that:
 - a) Different curves were obtained for the acid dyes, ponceau 2R and orange G, from those obtained with the basic dyes, toluidine blue O, methylene blue, Nile blue sulfate, and crystal violet.
 - b) When the concentrations of ponceau 2R and toluidine blue O were varied, the intensity of staining at any pH was a direct function of the concentration of dye.
 - c) When staining in toluidine blue O and ponceau 2R, stain intensity at a given pH-value was found to be a function of the buffer system in which the staining took place. "Isoelectric points" obtained by superposition of the two dye curves were higher in Sörensen's buffers (sodium citrate and HCl) than in McIlvaine's (citric acid and secondary sodium phosphate), and still higher in Clark and Lubs' (potassium acid phthalate and NaOH).
 - d) When the concentration of buffer was increased, a lower intensity of staining was obtained than with a more dilute buffer.
2. Various factors modifying staining intensity are discussed from a theoretical point of view. In the staining of a protein in buffered solution, it was shown that staining intensity (the index of the concentration of the dye-protein compound) at a given pH-value is dependent upon the interaction of the dye-protein, buffer-protein and dye-buffer systems, and that as the dye or buffer or their concentrations were varied, the resultant isoelectric points which were obtained also varied. In view of the present lack of knowledge of dyes and dye-protein combinations it would be impossible to determine a true isoelectric point by staining at controlled pH-values without further extensive work on the subject. It follows that the method cannot yet be applied to such a complex system as protoplasm, the aggregate of whose proteins have a more or less broad isoelectric range rather than a true isoelectric point, and which is by no means purely protein in nature.

3. No true isoelectric ranges have hitherto been obtained for nucleus, cytoplasm or other tissue elements by staining at controlled pH-values.

ACKNOWLEDGMENTS

I should like here to thank Dr. Roderick Craig for his helpful advice in the performance of the present research, Dr. Paul L. Kirk for furnishing the pure casein used in this research, and my wife, Helen Saxon Levine, for her assistance in the preparation of this paper.

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A METHOD FOR STAMPING SERIAL NUMBERS ON CELLOIDIN SECTIONS.

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One of the best methods of maintaining celloidin sections in serial order is the placement of numbers on the sections. This is a very useful procedure whenever removal of the celloidin is not necessary before mounting as it is relatively simple by comparison with other methods.¹ Furthermore, it allows storing and staining of the sections in bulk without loss of serial identity.

This method as first used by Suzuki² and later by Da Fano³ involves writing serial numbers on the celloidin sections with a fine pointed brush after first cutting and arranging them in sequence on a flat surface. The former used India ink as a writing medium while the latter added ether and acetone to improve the writing quality of the ink.

The procedure to be described facilitates the numbering process by simply stamping the figures on the celloidin with an appropriate ink prior to cutting the sections. This eliminates the task of arranging sections in sequence before marking and in addition the numeration is executed more efficiently with a stamp than a brush. During the past several years more than 10,000 sections of Weigert and Nissl preparations have been handled successfully in this manner.

Success depends largely on employing a stamp ink which is readily absorbed by the celloidin, dries quickly and sufficiently resists fading by alcohols and other reagents. A commercial product bearing the trade name of Triune Opaque Stamp Ink (black)⁴ has been found to satisfy these requirements. Rotating rubber number stamps to fit most any need may be procured thru a local stamp dealer. We have,

¹Reference is made to general accepted methods such as described in Mallory's Pathological Technique, pp. 61-64. Saunders, 1938.

²Suzuki, B. 1909. Eine einfache Schnittserienmethode bei der Celloidineinbettung. Anat. Anz., 34, 358-61.

³Da Fano, C. 1925. On the numbering in series of sections from celloidin blocks. Proc. Physiol. Soc., J. Physiol., 60, 13.

⁴Triune and Trojan stamp inks are manufactured by the Superior Type Company, 1900 W. Larchmont Avenue, Chicago, Ill. In case this product cannot be obtained locally the company will supply the name of the nearest dealer upon request. The usual price for a two oz. bottle is 55 cents. There may be similar inks made by other companies which would serve equally well.

however, used a dime store stamp after first removing the year and month bands. This provides numbers 1 to 99 which suffices in most cases since even large series of over 2,000 sections can be conveniently handled in groups of 99 or less.

The ink dries rather rapidly; therefore, it cannot be used on ordinary stamp pads in the same manner as the slower drying inks. The manufacturer recommends a special one (Opaque Stamp Pad) which reduces evaporation and consequent gumminess. A regular (uninked) pad, however, may be substituted if repeated small quantities of ink are applied as needed.

Method: An adequate margin of celloidin must be provided for placement of the numbers while embedding and trimming the block. Good imprints are obtained by keeping the stamp clean and applying the proper amount of ink and pressure. The numbered section is unrolled on the knife just before completing the cut. After completing the cut, the section is floated on water (contained in a wide shallow dish) with the inked surface upward, thus allowing the ink to dry before storing in 70% alcohol. In case only a single section is to be placed on a slide the sections may be stained and mounted without regard to their sequence. If more than one section is to be mounted on a slide, the desired sections must be selected before mounting. The slides may be easily arranged in proper order after placing them against a white background to offer contrast to the numbers on the celloidin. The slides may then be given corresponding numbers and appropriate labels. It has been found desirable to write or stamp on the slides with Trojan⁴ ink as it has excellent writing and wearing qualities on glass.

A METHOD FOR INJECTING INSECT TRACHEAE PERMANENTLY

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ABSTRACT.—By the use of an alcohol insoluble dye (trypan blue), acetic acid, and a detergent ("Santomerase No. 3"), a resulting dye solution is obtained which will completely penetrate the tracheal system of an insect. The dye is injected by the use of a vacuum and by the pressure produced when the air is allowed to re-enter the dye vessel. The dye itself is permanently fixed in the tracheae by means of a fixing solution containing alcohol, acetic acid and barium chloride as its components. The material must be properly preserved after staining. It may be stored indefinitely in 70% alcohol, xylol, cedar oil or clove oil, depending on whether the material is to be used for sectioning or for whole mounts. Injected material may be sectioned in either celloidin or paraffin, or may be cleared and mounted *in toto*.

At present there are only two ways of studying the tracheal system of an insect. One is to cut open the live specimen and examine the air-filled tracheae. The other method is to inject a colored oil into the tracheal system to facilitate observation. In each case the material must be used immediately as both air and oil disperse from the tracheae rapidly. Photographs of uncleared and unmounted material are unsatisfactory, and as permanent staining was not possible prior to this study, drawings were necessarily the only permanent record of research done on the tracheal system.

In the method described below, the injected material may be stored an indefinite length of time after fixation in 70% alcohol, xylol, cedar oil or clove oil, depending on whether the material is to be used for sectioning or for whole mounts. Drawings are not necessary as photographs may easily be made of the slides, which are themselves a fixed record.

To inject insect tracheae permanently the following solutions are required:

Solution A—the dye solution,

| | |
|--------------------------------------|--------|
| Trypan blue ¹ | 2.0g. |
| Santomerase No. 3 ² | 1.0g. |
| Glacial acetic acid..... | 10 cc. |
| Distilled water..... | 90 cc. |

¹Trypan Blue—National Aniline and Chemical Co. Inc. C. I. No. 477. Lot No. 7286.

²Santomerase No. 3—A detergent produced by the Monsanto Chemical Co., St. Louis, Mo.

Solution B—the fixing solution,

Formaldehyde (40%) 15 cc.

Glacial acetic acid 10 cc.

Saturated solution of barium chloride in distilled water 75 cc.

Live specimens are chloroformed and placed in a small wire basket. The basket is suspended above the stain by means of a fine wire. This wire is inserted into the aperture of the stopcock, which is then

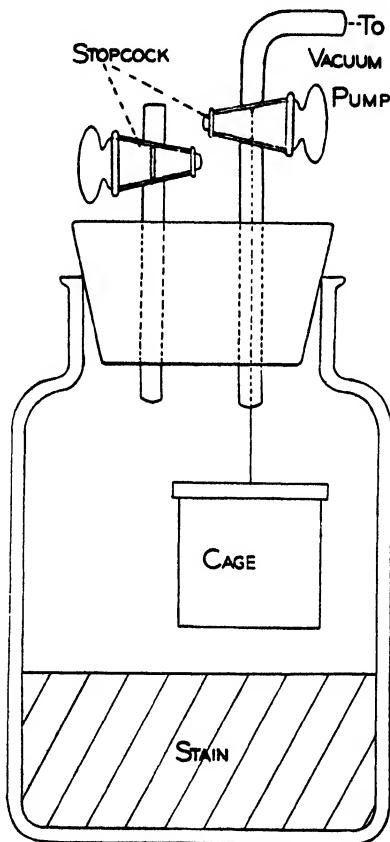


Fig. 1. Diagram of apparatus used to inject stain into insect material.

half closed, pinching the wire and thus holding the basket in place. The air is then exhausted from the vessel containing the stain by means of a vacuum pump. If the water pressure is good a faucet suction pump may be used to create the vacuum. In either case check must be made on the reduction of pressure. The manometer reading should in no case be less than 29 in. of mercury.

After 15 or 20 minutes the basket is dropped into the liquid by opening the stopcock and releasing the wire. The vacuum is maintained for 5 minutes longer and then the air is allowed to re-enter

the vessel slowly thru the other stopcock. (Fig. 1). The specimens are left submerged in the solution until the dye has completely penetrated the tracheae. This requires about 15 minutes.

Upon removal from the dye solution the material is fixed in solution B. Large insects may be fixed more rapidly by injecting the fixing solution directly into the body cavity with a hypodermic



Fig. 2. Photomicrograph of a portion of a celloidin section of the brain of the roach, *Periplaneta americana*, showing the extent of penetration of the stain when injected by the method described. Thickness— 20μ , Magnification— $\times 500$.

needle. This also distends the body sufficiently to prevent distortion caused by muscle contraction.

The specimens should be left in the fixing solution from 3 hours to over night depending on their size. They are then washed briefly in water and upgraded in alcohol to 70% by the usual method for histological material. As the dye is insoluble in alcohol, the stained material may be left in 70% alcohol indefinitely.

Sections may be made either by the celloidin or paraffin method. Safranin makes an excellent counterstain.

Examination of a celloidin section of the brain of the roach, *Periplaneta americana* (Fig. 2), shows the extent of penetration. The tracheal tubes may be traced, under oil immersion, to a point where they measure only 0.2μ in diameter. This demonstrates that all the tracheae and many of the tracheoles are stained.

The use of this method is limited to those forms which have the holopneustic or hemipneustic type of tracheal system. For this reason many of the larval forms of *Diptera*, *Neroptera* and *Odonata* can not be injected by this method.

NOTES ON TECHNIC

A SIMPLE METHOD FOR MOUNTING EMBRYOLOGICAL MATERIAL. During the course of some experiments conducted by the author for the past 2 years on the demonstration of centers of ossification in various mammalian embryos and fetuses, it became increasingly evident that a suitable method for mounting the specimens for permanent preservation was needed.

The well-known clearing methods utilizing KOH and glycerin were used, together with the respective staining methods for either bone or cartilage preparations. The final process of clearing and preserving was accomplished in pure glycerin (U. S. P.). Since the embryos varied greatly in size (in terms of crown-rump measurement) it was necessary to cut various sizes of glass plates to mount the finished specimens. This proved to be expensive and impractical, especially for material whose crown-rump measurements were less than 30 mm. Resort was finally made to the following procedure:

Sheet celluloid (as used in the automobile industry) was substituted for the glass slides or plates. The convenience of cutting the sheet celluloid to any desirable size and yet have a clarity equal to glass was soon realized. If, for example, an embryo whose crown-rump measurement was 45 mm. was to be mounted in glycerin in a museum jar, it was only necessary to cut the celluloid strip a bit larger and as wide as was needed. By tying the embryo to the celluloid support with the use of thread encircling the body, it was found to hold the specimen in a sturdy but natural position.

In a larger fetus, notches cut in convenient positions on opposite sides of the celluloid mounting strip afforded an adequate support for the specimens.

Buoyancy due to the density of the glycerin is eliminated after a day or so by penetration of the mounting medium into the specimen.

—CLARENCE W. NICHOLS, JR., Santa Cruz, California.

OLD GRUEBLER HEMATOXYLIN AND EOSIN COMPARED WITH CURRENT AMERICAN STAINS.—It was the recent good fortune of the writer to be given two bottles of stains marketed by Dr. G. Gruebler & Co. of Leipzig and imported by a Pittsburgh physician sometime before 1908 when they were stored in his attic. As the parchment covers of the brown glass bottles had not been opened the stains appeared to be in perfect condition. One bottle contained 300 grams of "Haematoxylin pur. cryst." and the other an equal amount of "Eosin w. gelblich."

Special interest attaches to these samples because they were purchased during Dr. Gruebler's lifetime and not many years after he left the business. At that time "Dr. Gruebler's Laboratory" (which subsequently became K. Hollborn and Sons) and "Dr. G. Gruebler and Co.", altho existing as separate concerns, were in cooperation¹. Because of the high repute of the Gruebler stains of those days, a comparison of these with current American stains appeared to be desirable.

The hematoxylin was examined in comparison with a sample of "Hematoxylin C.P." marketed by the Coleman and Bell Co. The Gruebler stain is coarsely granular, the particles varying in size from a powder to masses nearly a centimeter in diameter. In making the traditional .5% solution of hematoxylin the Gruebler stain dissolved more slowly in 95% alcohol, requiring 3 hours for complete solution to 1½ hours for the American stain. The straw color of the alcoholic solutions and of the diluted aqueous solutions was practically identical. On well fixed tissues, using the stains according to Heidenhain's iron hematoxylin method, a definite black staining of the chromatin and of chromosomes was obtained without obscuring coloration of the cytoplasm. The two stains also gave solutions of like color and staining quality when used as Delafield's hematoxylin solution. The results were so nearly identical as to indicate that the two stains are of equivalent staining quality.

The eosin stain is a fine powder similar in appearance to a sample of eosin Y, certification No. NE-4, of the National Aniline and Chemical Company. The Gruebler stain has about the same solubility in water and in 95% alcohol as the "water and alcohol soluble" American stain and the solutions are identical in appearance. Either in an aqueous solution or in an alcoholic solution the stains behaved similarly suggesting near equivalence in quality.

On the basis of these trials it appears that those who use certified American stains today have the equal in reliability of the famous Gruebler² stains of the first decade of the century.—T. M. McMILLION, Geneva Col., Beaver Falls, Pa.

¹The writer is indebted to Dr. H. J. Conn for supplying information about the history of the German firms.

²Samples of the Gruebler hematoxylin and eosin mentioned have been added to the collection of the Biological Stain Commission. Investigators who wish samples of the stains for comparative studies may obtain them from the writer on condition that the results of trials be reported to the Stain Commission.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

JOHANSEN, DONALD A. *Plant Microtechnique.* 6x9 in. 523 pp. Cloth.
110 illustrations. McGraw-Hill Book Co., Inc. New York, N. Y. 1940.
\$4.50.

This book, with about 500 pages of text, is perhaps the largest publication that has yet appeared in this country dealing exclusively with the field of botanical microtechnic. It is very attractively put up and illustrated largely from photographs of microscopic material prepared by the author. As he has spent much of his time in recent years preparing and marketing microscopic specimens, the material from which these photographs are prepared is excellent, and his experience with the microtechnical methods involved qualifies him to write on the subject.

The book is divided into two sections: the first of approximately 200 pages on "General Methods", and the second, of about 300 pages, entitled "Special Methods for the Various Phyla". The first of these two sections is a very adequate presentation of the methods employed in plant microtechnic with formulae of the most important solutions used in these methods; two of the sixteen chapters are devoted to stains and staining procedures. The second section of the book contains fifteen chapters, each dealing with special methods needed in the study of one of the large divisions of plants. Altho this is a somewhat unusual method of presenting the subject, it should prove useful to the student in this field.—*H. J. Conn.*

MICROSCOPE AND OTHER APPARATUS

BORRIES, B. v., and RUSKA, E. *Aufbau und Leistung des Siemens-Übermikroskopos.* *Zts. wiss. Mikr.*, 56, 317. 1939.

This article presents a concise, informative description of the new Siemens' supermicroscope compared with his two earlier models built by the same firm. This instrument is about as compact as a modern X-ray machine and but slightly more difficult to operate. Two photographs of the apparatus with diagrams comparing the conventional and electron microscopes, together with the description of the construction and operation of the latter, are included.—*J. M. Thuringer.*

MASON, W. *Apparatus for cutting frozen sections on the rocking microtome.* *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 86-8. 1939.

An apparatus is described for freezing sections which does away with the necessity for a freezing microtome. The object-holder of the rocking microtome is modified to allow of the application of CO₂ by cutting away the jaws into which the wooden blocks are clamped. Upon the resulting flat top and directly over the hole in the center is soldered a metal cap about 3 cm. in diameter by 1.5 cm. deep with holes in the sides which allow CO₂ to escape. The object-holder is attached to the CO₂ cylinder by means of a brass rod 3.5 cm. long, having an external diameter such that it just fits the shaft of the object-holder. A hole 3 mm. in diameter is drilled thru the center of the rod, and the lower part is threaded to fit a brass nut having the same size thread as the CO₂ cylinder. If the gas is applied in a steady gentle stream, the interior of the cap fills with CO₂ snow and the tissue will remain frozen long enough for sectioning.—*Jean E. Conn.*

REESE, J. D. A useful apparatus for staining slides. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 88-5. 1939.

An apparatus has been designed for staining slides which enables one to adjust the rack to a horizontal position and permits the dye to be poured back into the bottle without waste. A ring stand is used which has an upright bent at right angles about 2 in. from the end. A clamp holder is attached to the bent portion. The slide holder is made of stout brass wire, rectangular in shape, with a short metal rod attached to one end. By placing the rod in the free arm of the clamp holder, the rack may be easily adjusted to the horizontal. Small knobs of solder placed at intervals of about an inch along the rack keep the slides apart. A piece of monel metal with small metal "stops" opposite the spaces for the slides is soldered to one side of the rack in a slightly inclined position. When the rack is tilted, this piece of metal acts as a drain allowing the dye to flow down the incline into a bottle.—*Jean E. Conn.*

PHOTOMICROGRAPHY

KROGH-CHRISTOFFERSEN, A. Das Mikrophotographieren ohne photographische Geräte. *Zts. wiss. Mikr.*, 56, 301. 1939.

Photomicrography, without the use of cameras, by projection of the microscopic image directly upon suitable projection paper is described. A contact print from the paper negative obtained produces the finished picture. Any one interested in this method is referred to articles on the well-known paper negative processes.—*J. M. Thuringer.*

SMITH, HILTON A. A technique for making photomicrographic prints in color. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 45-51. 1939.

The author describes a method of adapting Eastman's "Wash-off Relief Process" to photomicrography. Since most sections are stained only in two colors, they can be reproduced by using only two negatives instead of three as is usually required. This method is described for tissues stained with hematoxylin and eosin, altho it may be adapted for other stains by using different filters. The A negative is exposed with the red light of two Wratten filters in combination, B (No. 58) plus E (No. 22). The corresponding positive is printed in the complementary color, a shade of blue, and reproduces the hematoxylin-stained nuclei. The red eosin is reproduced by the B negative which is exposed thru green filters. The writer found B (No. 58) plus H (No. 45) to be satisfactory; the complementary color, used in printing, is of eosin shade. The type of film preferred by the author is Process Panchromatic for the A negative and Panatomic for the B negative.

Chromatic aberration may be prevented by making a slight change in length of camera bellows between the two exposures. With a Bausch and Lomb type H camera, a 16 mm. apochromatic objective and an 8X compensating eyepiece, the bellows will need to be about 1.5 mm. shorter for the B picture than for the A. The exact amount, however, must be determined by experiment.

In selecting dyes, Eastman's "A" dye (blue) was found satisfactory, but not the "B" dye. To replace the latter, the writer uses a mixture of orange G (C. I. No. 27), 2 vol., Bordeaux red (C. I. No. 88), 1 vol., and amaranth (C. I. No. 184), 1 vol. The total mixture is used in a 0.3% aq. solution.

For further details the author refers to publications of the Eastman Kodak Co.
—*Jean E. Conn.*

DYES AND THEIR BIOLOGICAL USES

HÖBER, R. and BRISCOE-WOOLLEY, P. M. Conditions determining the selective secretion of dyestuffs by the isolated frog kidney. *J. Cellular & Comp. Physiol.*, 15, 35. 1940.

The influence of the molecular configuration of a number of sulfonic acid dyes of the mono-azo, dis-azo and triphenylmethane groups on their secretion by the proximal tubules of the isolated Ringer-perfused frog kidney has been studied.

The sulfonate derivatives of mono-azo and dis-azo dyes are secreted by the kidney tubules and are found in the secretion in a concentration higher than that injected into the renal portal vein, provided that the sulfonate groups are arranged in a special manner. The triphenylmethane dyes do not undergo any secretory transport across the kidney tubules. Lipoid solubility of the dyes favored their secretion but was not indispensable.

From the data it appears that for active secretion of the dyes to occur, a bilateral elongated molecule, with the sulfonate groups on one half of the molecule, is necessary, whereas if sulfonate groups are located at the two ends of the molecule, the secretion of such dyestuffs is prevented. It is suggested that the first stage in the process of secretion is the anchoring of the dye on the cell surface by the organophilic non-polar portion of the molecule, the hydrophilic polar part (containing the sulfonate groups) attaching at the aq. surface.—*L. Farber.*

KROLL, H., STRAUSS, S. F., and NECHELES, H. Concentration and detection of a dye in abscesses. *Proc. Soc. Exp. Biol. & Med.*, 43, 228-34. 1940.

To 100 mg. of an acid dis-azo dye, T 1824 (Evan's blue), in 15 cc. of water, aq. bromine was added, drop by drop, until the theoretical amount (2, 3 or 4 atoms) had been taken up. This was injected intravenously into dogs having abscesses, produced within 24 hr. after the injection of bacteria and foreign material. The abscess tissue was analyzed 20 hr. later. Best results were secured with mono-brominated dye. Fixation of the dye by abscess tissue was regarded as due to absorption on the cell membrane and retention by solution of dye in the lipoid material of the cell wall.—*M. S. Marshall.*

LISON, L., and FAUTREZ, J. L'étude physicochimique des colorants dans ses applications biologiques.—Etude critique. *Protoplasma*, 33, 116-51. 1939.

This is a critical analysis of the way in which many biologists have used dyes with little or no understanding of the varied properties of the dyes and have drawn conclusions which tend to be erroneous. An example given is the use of dyes in testing the lipoid nature of cell membranes by comparing the penetrability of the dyes with their solubility in fatty materials, without appreciating the variability of dye solubility in different fats and oils. There are also the investigations on the ultra filter theory by comparing intracellular penetration with the diffusibility of the dyes in solution without considering the nature of the diffusion medium. The authors stress the importance of understanding the physicochemical properties of the dyes, especially in biological media.

The length of the article limits a description to an enumeration of the chapters as follows: (1) Nomenclature and classification; (2) Chemical reactions and transformations, e.g., reducibility, formation of basic carbinols (pseudobases) and metachromasia; (3) Diffusibility and dispersibility in water, gels and dialysis; (4) Liposolubility, varying according to the lipoid material used and the possibility of hydrophily of some lipoids; (5) Electric charges; (6) Flocculability; (7) Electrocapillarity and surface activity (tensio-activité); (8) Physicochemical modifications in colloidal media, especially in serous media. A list of over 100 references is appended.—*Robert Chambers.*

MIRIMANOFF, A. Remarques sur la sécretion des tentacules de Droséra. Notes histochimiques. *Protoplasma*, 33, 211-14. 1939.

The author tested the reaction of the secretion to various agents by several methods, the best of which he found to be the following. Filter paper was moistened with a given reagent, then dried and a leaf of *Drosera* placed on the filter paper so that only the tips of the tentacles were in contact with the filter paper. Any reaction which took place at the points of contact was considered to be the result of the secretion at the tips of the tentacles. The reagents used were silver nitrate, parachlor-indophenol, o-nitroso-nitro benzol, ferric chloride, sodium nitro-prussate, Fehling's solution and potassium ferricyanide with ferric chloride. All showed the presence of a reducing substance. The results obtained convinced the author that the secretion contains ascorbic acid.—*Robert Chambers.*

ANIMAL MICROTECHNIC

COOKE, JEAN V., and BLATTNER, RUSSELL J. Vital staining of virus lesions on chorio-allantoic membranes by trypan blue. *Proc. Soc. Exp. Biol. & Med.*, 43, 255-6. 1940.

One cc. of trypan blue, 0.5% aq., is placed on the membrane of the infected embryo; the egg is gently rotated and incubated 10-30 min. The membrane is removed, washed in saline and fixed flat in 10% formalin for a few minutes. The membrane is then drained, flattened on a 2x2 in. slide and mounted in glycerin gelatin, (50% glycerin, 5% gelatin, 1% phenol). Mounting is done at 70° C. The edges are sealed with balsam or asphalt.—*M. S. Marshall.*

INGLEBY, HELEN, and HOLLY, CLAIRE. A method for the preparation of serial slices of the breast. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 98-6. 1939.

The following method is described for making serial sections from whole breasts: Fix breast by suspending it in a gauze sling in a jar of 4% formaldehyde; leave jar at least 24 hr. in the refrigerator room; remove breast from solution, wrap in a wet cloth and freeze. (The commerical ice cream freezer is handy for rapid freezing. The slicer put out by the American Slicing Machine Co. is the most satisfactory for making the serial slices.) After slicing, pile sections in order. (Difficult sections may be received in a bowl of water, floated onto numbered pieces of paper and stacked.) Fix sections a few hours; rinse in dist. water; stain 24 hr. in a single layer in a filtered solution of 5 cc. Harris' hematoxylin in 100 cc. dist. water; turn once to insure even staining; differentiate in acid alcohol; wash until blue; 70% alcohol, 1-2 hr.; 95% alcohol, 6-8 hr. (2 changes); abs. alcohol, 2-4 hr. (2 changes); benzine, 2-4 hr. (2 changes); store sections in mineral oil until required for use. Then transfer to oil of wintergreen (2 vol.) and benzol benzoate (1 vol.) for about 1 hr. before examining under the dissecting microscope. Masson's iron hematoxylin is an alternative stain recommended if photography is contemplated. After study sections must be returned to mineral oil.

The advantages of this method are: Every part of the tissue may be examined grossly; a three dimensional view is obtained; a whole breast can be ready for examination in 5 days; paraffin sections can be made at any time from any part if higher magnification is desired.—*Jean E. Conn.*

JALOWY, BOLESLAW, and CHRZANOWSKI, BRONISLAW. Einige Bemerkungen über den Vorversilberungsprozess. *Zts. wiss. Mikr.*, 56, 384. 1939.

The authors review the theories advanced concerning the action of silver salts in impregnation processes (primary and secondary impregnations). Their work deals with the action during primary impregnation of tissues (preargentation as distinguished from postargentation when the silver salts are applied after preliminary treatment with suitable fixative or mordant). They conclude, on the basis of their investigation, that during preargentation the chlorine ions play the exclusive role in forming light sensitive AgCl.

They demonstrate that chlorine ions could be rapidly removed from the mesentery and other tissues by rinsing in large quantities of dist. water or by previous fixation with 10% or 20% formalin.

The AgCl formed during preargentation is converted into metallic silver under the action of light. This process does not begin spontaneously and when inaugurated thru light does not progress further. Pure silver albuminates do not darken with the action of light; their role is limited to the formation of a stroma (substrate) in which the granules of metallic silver are deposited. Silver phosphate, ortho-, meta-, and pyro-phosphate are likewise insensitive to light.—*J. M. Thuringer.*

KRAMER, FRANK M. Macroscopic staining of anatomic and pathologic specimens. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 72-8. 1939.

Methods are described for the macroscopic staining of fat, iron, calcium, amyloid, and the brain. The following method is recommended for staining fat: Fix in formalin; cut a fresh surface and immerse in a sat. solution of Sudan III

or "scharlach R" (i.e. Sudan IV) in 70% alcohol until the fat has assumed a brilliant red color; avoid overstaining; decolorize in 95% alcohol for 12 hr. or more; wash thoroly in running water; mount in weak formalin. Fat-products of degeneration are also stained, but normal fat is stained more intensely.

For iron, the Berlin blue method is recommended as follows: Place specimen in a freshly prepared solution of equal parts 2% $K_4Fe(CN)_6$ and 1% HCl, until the desired reaction takes place; wash; preserve or mount in 70% alcohol.

The following silver nitrate method is recommended for staining calcium: Wash formalin-fixed specimen at least 24 hr. in running tap water; process thru several changes of dist. water for about 24 hr.; stain in the dark for 6-15 hr. in 1% $AgNO_3$ in dist. water; rinse in dist. water without exposing to light; place for a full day in 5% hypo solution; wash thoroly; mount in 50% alcohol or Kaiserling's preservative. Calcium may be stained a vivid pink color by alizarin dyes as follows: Stain 12 hr. in a 1:10,000 solution of alizarin red S, made basic by adding a small amount of KOH; differentiate for several days in equal parts of alcohol and glycerin, exposing jar to sunlight; mount in an alkaline preservative, e.g. Kaiserling's with a small amount of KOH added (1:1000).

Immersion in Lugol's iodine solution stains amyloid an intense brown, made sharper by adding weak H_2SO_4 . To prevent fading, equal parts of 2% Lugol's and 1% H_2SO_4 are used as preservative.

The best method for staining the brain is as follows: To prevent tinting of the white matter, first process the specimen for a few min. in a phenol solution (phenol crystals, 80 g.; HCl, 3 cc.; $CuSO_4$, 10 g.; dist. water, 2000 cc.); immerse in cold tap water; stain in 0.5% nigrosin solution; rinse and examine occasionally to determine progress of staining; when the correct intensity is reached, remove "excess" dye with weak alcohol; rinse in running water; mount in weak formalin. This is the only method in which the intensity of staining can be favorably controlled.—*Jean E. Conn.*

LENDRUM, A. C., and MCFARLANE, D. A controllable modification of Mallory's trichromic staining method. *J. Path. & Bact.*, 50, 381-4. 1940.

By applying the stains of the Mallory technic one at a time the effects desired may be attained with greater assurance. For the nuclear stain the following procedure is used: Allow 2.5 g. iron alum to dissolve over night at room temp. in 50 cc. dist. water. Add to this 0.25 g. celestin blue (C. I. No. 900) and boil the mixture 3 min. Filter when cool, and add 7 cc. glycerol. Stain in this solution 10-20 min. Rinse in water. Add filtered Mayer's hemalum; stain 5-10 min. Rinse in 95% ethanol. Differentiate in acid alcohol (1% HCl in 95% ethanol) until the red color ceases to come off.

After this step, the cytoplasm is stained as follows: Treat with 0.2% orange G in 80% ethanol saturated with picric acid, 2 min. to 16 hr. Rinse in water 30 sec. to 2 min. From filter add fuchsin-ponceau solution (prepared by mixing 1% acid fuchsin in 1% acetic acid with 1% ponceau 2R, C. I. 79, in 1% acetic, and adding 2.5 cc. of 10% Na_2SO_4 to each 100 cc. of dye solution to inhibit mold growth). Stain for 15 sec. to several min. Rinse in 1% acetic acid and examine. Decolorize connective tissue in 1% phosphomolybdic acid; do not decolorize completely. Tissue fixed without chromate is sufficiently decolorized in 1-2 min. Stain in 2% soluble blue (C. I. 706) in 1% acetic acid, 2-10 min.; or in fast green F.C.F. in 1% acetic, 2-10 min. Rinse in 1% acetic acid and examine; if satisfactory, dehydrate rapidly and mount.

By this technic the nuclei stain dark red and the blood is colored distinctly different from the fibrin. This nuclear stain is more resistant to the action of picric acid than iron alum hematoxylin. The celestin blue solution should be replaced every 6 months; the other stain solutions last longer. The nuclear stain may appear to be entirely removed in the acid alcohol, but a mordanting effect is achieved in the nucleus that is not affected by picric acid and which fixes the acid fuchsin. Excess fuchsin staining may be remedied by immersion in the picric-orange-G solution. To emphasize color differences for photography, sections may be mordanted before staining in Hollande's solution without acetic acid. Poorly stained or faded sections can be re-stained by immersion in picric orange G or in a saturated solution of picric acid in 80-100% alcohol for 30-60 min., then rinsed and restained. Biebrich scarlet can replace the fuchsin-ponceau mixture. "Revector" dyes were used by the writer.—*S. H. Hutner.*

LILLIE, R. D. The effect of hydrogen-ion concentration of formaldehyde used in storage for varying periods on staining of tissue. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 89-92. 1939.

The author reports experiments which show that formaldehyde solutions buffered to pH 7.5 preserve staining reactions of tissues for Romanowsky staining in particular and for nuclear staining in general much better than does formaldehyde not so buffered. The need for further work on this point is indicated.—Jean E. Conn.

MICHAEL, E. G. Rapid method of staining frozen sections of tissues requiring immediate diagnosis. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 47-9. 1940.

The writer recommends the following rapid technic: Put tissue in 20 cc. of 10% formalin, boil 1 min. Cut sections and put in dist. water in a Petri dish. Put a section on a slide in dist. water in another Petri dish. Blot, cover with celloidin in alcohol-ether, and blot again. Put in dist. water, 20 sec. Remove. Cover with filtered Delafield's or Ehrlich's hematoxylin, 20 sec. Rinse in dist. water. Cover with 1% eosin Y in 95% alcohol, 15 sec. Add 3 drops of Mallinckrodt's new beechwood creosote without removing the eosin. As soon as it penetrates, add 1 drop of Canada balsam in xylol and apply a cover slip. Wipe off excess mixture of eosin, creosote and balsam with a piece of gauze moistened with xylol.—G. H. Chapman.

ÖKLAND, FRIDTHOF. Untersuchungen über Osteoblasten in Schuppen und Ausstrichen. *Zts. wiss. Mikr.*, 56, 845. 1939.

This work is based on the Rupprecht and Krompecher methods of preparing ground bone sections with combined fixation and staining. The author suggests the following modifications. Thin pieces of cranial vault of mouse, rat, or guinea pig (5 mm. sq.) are fixed for 24 hr. each in 60%, 95%, and abs. alcohol, and xylol. They are then ground on small carborundum hones saturated and kept wet with xylol. The thin sections are rinsed in clean xylol and brought into abs. alcohol, 5 min.; methyl-green-pyronin (Pappenheim, Grüber), 24 hr.; abs. alcohol, 10 min.; xylol, 1 hr.; neutral balsam. Results may be varied by previous *in toto* staining with Nile blue sulphate (sat. solutions in 60%, 95%, and abs. alcohol), then proceeding as above including the methyl-green-pyronin stain after grinding sections.

Bone marrow smears for control preparations may be stained with May-Gruenwald (similar to Wright's) or in the following way: a) methyl-green-pyronin, 3-5 min.; b) rinse in dist. water; c) abs. alcohol, xylol, and neutral balsam.

Results: cytoplasm stains red; characteristic vacuole, colorless.—J. M. Thuringer.

PARMENTER, C. L. Chromosome numbers in *Rana fusca* parthenogenetically developed from eggs with known polar body and cleavage histories. *J. Morph. and Physiol.*, 66, 241-60. 1940.

Modifications of Bouin's solution (B-8 and B-15 without urea) are used in fixation. Since alcohols shrink the jelly into an unremovable, thin, tough coat, the eggs should be washed and preserved in 4% formalin, with frequent additions of Li₂CO₃ during washing. The following procedure is recommended: Before embedding, place eggs in 35% alcohol and dissect jelly from the eggs with very fine insect needles. Dehydrate and clear eggs in 30-min. successive baths of $\frac{1}{3}$ dioxan, $\frac{2}{3}$ dioxan, and 2 changes of pure dioxan. Infiltrate successively in equal parts of dioxan and soft paraffin at 47° C., soft paraffin at 47° C., and hard paraffin or tissuemat at 56° C. for about 15 min. each. Section eggs at 18 μ , with the knife passing thru both poles simultaneously. Of several stains used, Heidenhain's hematoxylin without counterstain, and safranin followed by light green or fast green give the most satisfactory results.—Elbert C. Cole.

PERRY, I. H., and LOCHHEAD, M. S. Histological technique for the pituitary gland of the mouse. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 101-3. 1939.

A reliable differential stain for the pituitary of the mouse is described as follows: Remove the gland as rapidly as possible from the anesthetized animal by reflecting back the skin over the head and cutting the skull from its base. Cut out a wedge of bone around the pituitary and put into the fixing solution without handling. After hardening 15-30 min., dissect from the bone with needles under a binocular microscope without removing from the fixative. Transfer the pituitary with a pipette.

Fixation and embedding: Fix 4 hr. in Zenker-formaldehyde (95 cc. Zenker plus 5 cc. neutral 39% formaldehyde). Add the formaldehyde to the Zenker immediately before use). Wash 4 hr. in dist. water (many changes); dehydrate in 50% and 60% alcohol for ½ hr. each; leave over night in 70% alcohol; 70% iodized alcohol, 1 or 2 hr.; 80%, 90% and 100% alcohol, 2 hr. each; ether-alcohol, 2 or 3 hr. at 36° C.; one day each in 10%, 25% and 50% nitrocellulose, or 2%, 4%, 6%, 8% and 16% celloidin; harden, and cut at 4 μ .

The slides are stained using the technic described by Koneff (Stain Techn., 13, 49-52, 1938) with the following modifications: Before staining, leave 12 hr. in 3% potassium bichromate, and rinse in dist. water. In step 1 treat with anilin alcohol 18 hr. instead of 45 min. In step 3 leave in azocarmine solution 4 hr. at 56° C. and 14 hr. at room temp.

The results are: Basophiles light blue with bluish-red nuclei; two types of acidophiles shown, one stained orange, the other deep red; nuclei of the acidophiles pink, bluish red or dark red; chromophobes gray with nuclei usually red; mitochondria in the basophiles always stained red.—Jean E. Conn.

SCHALM, O. W., and HARING, C. M. A technique for reducing soft-tissue organs to thin serial slices, with special reference to its use on bovine mammary glands. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 97-100. 1939.

A technic is described by which the entire bovine udder may be reduced to serial slices. The procedure is as follows: Remove the entire mammary gland at slaughter; milk out as much secretion as possible; inject the duct and vascular systems with 8% formaldehyde, using a 5-liter aspirator bottle fitted with a rubber bulb for raising the pressure in the bottle. Inject the vascular system first thru the mammary artery of each mammary half; ligate the stumps of the large vessels as the formaldehyde escapes; continue injection until all the vessel stumps are so closed. Inject the duct system of each quarter thru the teat canal until the quarter is distended and tense. A round wooden applicator may be inserted into the teat canal to avoid distortion during hardening. Suspend the entire udder in 4% formaldehyde until thoroly hardened (2-6 weeks); separate the mammary halves along the median line; trisect the halves by cutting thru the center of each quarter from the tip of the teat to the base of the gland; slice each piece in an electrically-driven meat-slicer, making the slices from 3-5 mm. in thickness; attach paraffin-coated tags to each slice for identification; wrap together in cheese-cloth; store in 4% formaldehyde. The procedure may be suspended at any stage as long as the tissues are kept submerged in paraffin.

The thin slices may be dehydrated in a graded series of either dioxan or tertiary butyl alcohol and infiltrated with paraffin. The slices are cooled between two glass plates, excess paraffin removed by gentle heat, and a protective coat of white shellac applied. Slices so prepared are best observed by transillumination.—Jean E. Conn.

VRAA-JENSEN, G. Eine Method zur Doppelfärbung von Übersichtspräparaten des Zentralnervensystems. *Zts. wiss. Mikr.*, 56, 956-8. 1939.

This method is suited for "survey sections" of the central nervous system with excellent contrast between myelinated fibers and nerve cells. Sections are cut in paraffin (15 μ) and celloidin (20-25 μ).

Technic: 1) Deparaffinize. 2) Dist. water, 2 changes. 3) Stain in iron hematoxylin at 50-55° C. (paraffin sections, 10-15 min., celloidin sections, 20-30

min.). Prepare by mixing equal parts of 4% aq. $\text{FeNH}_4(\text{SO}_4)_2$, and 1% hematoxylin solution (10 ml. ripened solution hematoxylin to 90 ml. dist. water) immediately before use. 4) Tap water, 2 changes. 5) Differentiate in 4% aq. $\text{FeNH}_4(\text{SO}_4)_2$ (paraffin sections until the blue color is no longer visible thru the under surface of the slide; celloidin sections until the celloidin is colorless). 6) Tap water, 3 changes. 7) Differentiate, controlling with microscope, until cells just lose their stain in the following solution: 10 g. sodium borate, 12.5 g. $\text{K}_3\text{Fe}(\text{CN})_6$ to one liter of dist. water. 8) Dist. water, 2 changes. 9) Rinse 5-10 min. in Vlesschouwer's pH 11 buffer solution (97.3 ml. of 0.53% aq. Na_2CO_3 , and 2.7 ml. of 1.91% aq. NaBO_3). 10) Stain paraffin sections, 4 hr., or celloidin sections, 2 hr. in the following solution: Dissolve 0.1 g. Kernechrot (Hollborn), in 100 ml. 5% $\text{Al}_2(\text{SO}_4)_3$ by heating slowly, stirring constantly, and bringing to boil for 5 min.; allow to cool to room temp. and filter; add sufficient water thru the filter to bring the filtrate to 100 ml.; for every 5 vol. of the stain add 1 vol. N/1 NaOH. 11) Dist. water, 2 changes. 12) Dehydrate thru xylol and mount in balsam.

Results: Myelin sheaths, dark blue; nerve cells, connective tissue, non-myelinated fibers, and nuclei of neuroglia stain red; nerve cells stain most brilliantly while the intensity of the other structures gradually diminishes in order given. In the cerebrum the best results are obtained with celloidin.—J. M. Thuringer.

PLANT MICROTECHNIC

TANAKA, N. Chromosome studies in Cyperaceae. VI. Pollen development and additional evidence for the compound chromosome in *Scirpus lacustris* L. *Cytologia*, 10, 348-362. 1940.

In connection with a cytological study of pollen development, the following recommendation is made:

After aceto-carmine stains, seal the cover-glass in place with melted "valap", a mixture of 2 parts vaseline, 2 parts lanolin, and 1 part paraffin, applied with a glass rod. If necessary, remove the valap with chloroform.—Virgene Kavanagh.

MICROÖRGANISMS

BRUNER, D. W., and EDWARDS, P. R. Application of the endospore stain to blood smears from opsonophagocytic tests. *J. Lab. & Clin. Med.*, 25, 543-4. 1940.

This stain permits an accurate count of phagocytosed bacteria. A smear is prepared from the test material as follows: Spread the contents of a 2 mm. loop over 4 sq. mm. of a slide; air dry; fix by flaming 3 times; apply 5% aq. malachite green, 5 min.; wash 10-20 sec. in tap water; apply 0.5% aq. safranin, 10 sec.; wash quickly with tap water; dry and examine. The method is not applicable to all species of bacteria, for some do not retain the green in the presence of the safranin. Staphylococci are stained red. Streptococci are stained red for the most part but a few cells stain green. Some Gram-negative bacilli are stained red and others green. This technic gives excellent contrast between bacteria and white blood cells.—John T. Myers.

CANSEY, O. R. Description of three species of frog microfilariae with notes on staining methods. *Amer. J. Hyg.*, 30, 117-21. 1939.

In the course of the writer's investigations of infected frogs, four stains for blood smears were employed: (1) Hemalum. Dried smears were dehemoglobinized in saline, placed in warm 70% alcohol for 15 min., passed thru tap water, then thru slightly alkalized water and placed in the stain for 3-5 hr. They were destained in 70% alcohol containing 1% HCl until differentiated. After washing in tap water they were passed thru 70, 80, 95 and 100% alcohol, cleared 5 min. in xylol and mounted in balsam. (2) Methyl-green-pyronin: 0.2 g. methyl green and 0.075 g. pyronin in 100 cc. of isotonic NaCl solution. After being dehemoglobinized in saline, smears were placed in the stain for 12-36 hr., washed and mounted. (3) Azure-II-eosin. A stock solution of azure II was prepared

by adding 1 cc. each of 1% aq. azure II and 1% aq. Na₂CO, to 500 cc. of saline; this was diluted in 24 vol. of saline for staining. Dehemoglobinized smears were stained in the dilute azure for 3-5 hr., removed, and without washing, several drops of 0.025% eosin in saline added. A cover-slip was sealed in place with vaseline. (4) Vital azure II. Fresh blood was placed on a slide on which several drops of the stock azure II of No. 3 had been dried. After mixing, a cover-slip was put in place, and examination was made while fresh.—John T Myers.

FITE, G. L. The fuchsin-formaldehyde method of staining acid-fast bacilli in paraffin sections. *J. Lab. & Clin. Med.*, 25, 748-4. 1940.

The most reliable means of staining acid-fast bacilli in sections is by the following method, washing in tap water between each step: Place in a solution, containing new fuchsin 0.5 g., phenol crystals 5.0 g., alcohol (methyl or ethyl) 10 cc. and water to make 100 cc., for 12-24 hr. at 60° C. or 24-48 hr. at room temp.; transfer to freshly dist. aq. 5-30% formaldehyde, 5 min.; place in alcohol containing 2% of HCl, 10 min.; 1% aq. KMnO₄ until brown, usually 2-5 min.; 2% aq. oxalic acid, 1 min.; stain 2 min. in Harris' hematoxylin; then in Van Giesen's stain (acid fuchsin 0.1 g., picric acid 0.5 g., dist. water to make 100 cc.). Without washing, dehydrate in alcohol, clear in xylol and mount in balsam. Nuclei stain brown, connective tissue fibers red, muscle fibers yellow, and acid fast bacilli dark ultramarine blue. Lepra bacilli impossible to demonstrate by other methods were readily stained.—John T. Myers.

HAKANSSON, E. G. A method of destroying the blastocysts (*Blastocystis hominis*) in fecal wet smears in order to facilitate the examination of Endamoeba histolytica. *J. Lab. & Clin. Med.*, 25, 546-7. 1940.

Blastocysts can be ruled out by making the fecal preparations in water instead of isotonic saline. Blastocysts and protozoon trophozoites will disintegrate but cysts will remain for several hours.—John T. Myers.

HORNUS, G. J. P. Psittacose pulmonaire expérimentale de la souris blanche. *Ann. Inst. Pasteur*, 64, 97-116. 1940.

The following procedure is recommended for staining elementary bodies concentrated from emulsified infected lung: Smear suspension on a slide; dry; fix with methyl alcohol; when nearly dry, rinse thoroly in tap water; stain in 1% toluidine blue. Rinse; differentiate in 1% eosin-orange (neither formula nor source of dye stated); rinse, dry and examine. Five min. in toluidine blue suffice for recognition of the elementary bodies; longer staining gives more intense colors. The bodies are violet blue.

A modified Dominici technic is described for staining elementary bodies in sections. Fix in Bouin; cut paraffin sections and bring down to water; mordant ½ hr. in Lugol's solution; decolorize with Na₂S₂O₃; rinse in water. Stain ¼ hr. in a mixture of 1% erythrosin, and 1% orange G. Wash in running water; stain 5 min. in 1% toluidine blue. Differentiate in 0.2% acetic acid until the sections are rose-colored. Dehydrate and mount. Protoplasm of the cells is rose, with nucleus violet and elementary bodies clear blue.

An alternative Giemsa method is as follows: Prepare sections after Bouin fixation; rinse in buffer (2% Na₂HPO₄, 1% KH₂PO₄). Stain in Giemsa solution freshly prepared by adding 25 drops of dye to 25 ml. neutral water. Stain 12 hr., then an additional 96 hr. in fresh stain solution. Wash in running water; differentiate in a mixture of 1% orange G and 5% tannic acid until the section has an orange tint. Change the latter solution once or twice as it becomes saturated with the Giemsa stain. The results are cytoplasm yellow, nuclei brown, and elementary bodies intense violet. A color plate illustrates these effects.—S. H. Hutner.

JOHNS, C. K., and HOWSON, R. K. Potentiometric studies with resazurin and methylene blue in milk. *J. Dairy Science*, 23, 295-302. 1940.

Coefficients of correlation of the reduction test times and the Breed counts of individual cells on 369 samples of market milk were $r = -0.711 \pm 0.017$ for the

resazurin test, using a definite pink color as the end-point, and $r = -0.651 \pm 0.020$ for the methylene blue test. The tubes were inverted every two hours in the reduction tests. The ratio of the resazurin reduction times to the methylene blue reduction times was approximately 3:4. The shapes of the time-potential curves obtained with the two dyes indicate that the shorter reduction time with resazurin is due to the more rapid initial drop in potential which occurs. Shortly below the point where the change to pink takes place, the curve flattens somewhat before again declining to reach the same final potential level as the milk to which methylene blue was added. In the range immediately following reduction to the pink, resazurin seems to have a greater poising action than does methylene blue at the same level of potential. More rapid reduction as a result of inversion of the tubes at hourly intervals is due to more active growth of bacteria. The resazurin test apparently is more satisfactory than the methylene blue test for grading samples containing considerable numbers of slow-reducing thermouduric organisms.—F. E. Nelson.

MATUSZEWSKI, T., and SUPINSKA, J. Studies on the methylene blue reduction test. II. Comparison between the old and the modified methods. *J. Dairy Research*, 11, 48-50. 1940.

Methylene blue reduction tests were made on each of 185 milk samples, using both the old method and the new method of Wilson, in which the tubes are inverted every half hour until reduction occurs. The new technic gave a shorter reduction time than the old, and smaller variations in the numbers of bacteria (determined by microscopic examination of stained preparations) corresponding to a given reduction time. The numbers of organisms actually present in the milks at reduction time approximated very closely the numbers calculated by means of equations whose derivation is presented. More bacteria were present at the time of reduction in the inverted tubes. Abnormally short reduction times, possibly due to aeration before the test began, and unusually long reduction times, apparently due to slowly active bacteria, were encountered at times. The authors believe the proposed modification constitutes an advance in the reduction test technic.—F. E. Nelson.

PARSONS, R. J. The staining of Negri bodies in formaldehyde and alcohol fixed tissues. *J. Techn. Methods & Bull. Int. Assoc. Med. Museums*, 19, 104-8. 1939.

The following procedure is described as a simple and reliable stain for Negri bodies in tissue fixed in formaldehyde or alcohol: Fix in 4% formaldehyde 18 hr. to 7 weeks or in 96% alcohol 18 hr. to 3 weeks. (It is preferable to dissect out the hippocampal gyrus before fixing and to prepare several cross-sectional blocks. The cerebellum should always be fixed.) Float paraffin sections cut at 4-5 μ directly onto clean cover slips or slides; dry over night in a warm oven or warm over a flame until the paraffin just begins to melt; remove paraffin from sections and bring to 96% alcohol. Stain 2 min. in acidulated ethyl eosin prepared as follows: Add 1.25 cc. of a solution of 1.0 g. ethyl eosin (National Aniline & Chemical Co., certified) dissolved in 100 cc. ethyl alcohol 96% and filtered before use to 22 cc. of a solution of 0.6 cc. acetic acid (Reagent Special, 99.5% acetic acid) in 99.4 cc. dist. water. Wash off excess stain in 96% ethyl alcohol, leaving sections a medium pink color. Stain 2 min. in borax-methylene-blue (methylene blue, 1.0 g.; borax, 1.0 g.; dist. water, 100 cc.). Rinse briefly in dist. water. Differentiate 2-5 min. in 0.25% acetic acid (99.5% acetic acid, 1.0 cc.; dist. water, 400.0 cc.), making sections a pale bluish-pink. Rinse in 96% alcohol; dehydrate as rapidly as possible in 2-3 changes of abs. alcohol; xylol (2 changes); mount in balsam.

Results: Negri bodies bright orange-red with a vacuolated inner structure or a small blue central dot; Lyssa bodies bright orange-red, but uniformly stained; nucleoli dark blue; red blood cells an intense copper red; nuclei blue; Nissl substance bright blue; cytoplasm of nerve cells pale pink; neuroglia pale pink. The differential coloration of the Negri bodies is so striking that they can be found under low ($\times 100$) magnification, using higher magnification only for positive confirmation. This stain has been employed successfully on material from dogs and man.—Jean E. Conn.

STEINER, GABRIEL. A simple method of staining the spirochaetes in routine paraffin sections. *J. Lab. & Clin. Med.*, 25, 204-10. 1939.

The following technic is proposed: Dehydrate tissue blocks in graded alcohols, clear in xylol, embed in paraffin, cut 9-10 μ thick and place in abs. alcohol (2 changes). Place sections in the following solution for 1-1½ min.: uranium nitrate (4% in abs. alcohol), 20 cc.; gum mastic (25% in abs. alcohol), 40-50 cc.; abs. alcohol, 20-30 cc. Wash in 8 changes of dist. water. Place in 0.1% aq. AgNO_3 for 1.0-1½ hr. at 100° C. Dehydrate in graded alcohols. Place in 10-12.5% gum mastic (in abs. alcohol) for 5 min. Wash 3 times in dist. water. Reduce for 5 min. in 5% hydroquinone (hydroquinone, 10 g.; 12.5% alcoholic gum mastic, 1 cc.; dist. water, 200 cc.) Counterstain, if desired, with hematoxylin and eosin, cresyl violet, or fuchsin. Dehydrate, clear and mount. The method is simple, rapid and free from precipitates.—*John T. Myers*.

TAYLOR, DEAN M. A study of procedures for detection of coliform organisms in Minnesota drinking water. *J. Amer. Water Works Assoc.*, 32, 98-104. 1940.

In order to find a method of detecting coliform organisms in water, less laborious and time consuming than the *Standard Methods* "completed test", 1,534 samples of drinking water were tested using (1) that method, (2) brilliant green lactose bile and (3) fuchsin lactose broth. The second compared very favorably with the first and might advantageously replace it. For isolating coliform organisms, the alternative "completed test" of *Standard Methods*, employing brilliant green lactose bile, was preferable to the usual "completed test".—*Merritt N. Pope*.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the April number of this Journal.

STAINS CERTIFIED MAR. 1, TO MAY 31, 1940*

| Name of dye | Certification No. of batch | Dye Content | Objects of tests made by Commission† | Date approved |
|-------------------|----------------------------|-------------|-----------------------------------------------------------------------------------|---------------|
| Nigrosin WS | CNi-3 | | For negative staining of bacteria | Mar. 18, 1940 |
| Chlorazol black E | NBc-1 | | As histological and cytological stain | Mar. 18, 1940 |
| Eosin Y | CE-10 | 92% | As histological stain and as a constituent of blood stains | Apr. 1, 1940 |
| Crystal violet | CC-10 | 90% | As histological, cytological, bacteriological stain, and in bacteriological media | Apr. 1, 1940 |
| Carmine | LCa-2 | | As histological and cytological stain | Apr. 4, 1940 |
| Carmine | NCa-7 | ... | As histological and cytological stain | Apr. 4, 1940 |
| Alizarin red S | LAr-2 | | As stain for bone | Apr. 10, 1940 |
| Congo red | NQ-8 | 87% | As histological counter-stain | May 10, 1940 |
| Nigrosin | NNi-7 | | For negative staining of bacteria | May 16, 1940 |
| Eosin Y | NE-15 | 87% | As histological stain and as constituent of blood stains | May 23, 1940 |
| Crystal violet | NC-21 | 90% | As histological, cytological, bacteriological stain, and in bacteriological media | May 24, 1940 |
| Brilliant green | NBg-9 | 94% | For use in bacteriological media | May 31, 1940 |

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

STAIN TECHNOLOGY

20. DEC. 1940

VOLUME 15

OCTOBER, 1940

NUMBER 4

LIBRARY

AN APPLICATION OF THE FROZEN SECTIONING TECHNIC FOR CUTTING SERIAL SECTIONS THRU THE BRAIN

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ABSTRACT.—The availability of CO₂ ice makes it practical to cut large blocks of cerebral tissue by the freezing method. If the tissue is first treated with 20–30% ethyl alcohol for sufficient time to secure uniform penetration of the alcohol (about 24 hours), formation of hard ice crystals can be controlled and serial sections 25–100 μ thick can be cut with negligible loss. The alcohol can be added to the fixative used for perfusion, or it can be added at any time later in the fixing process, or after fixation is completed. The sections are cemented to the slide and groups of slides are manipulated thru staining processes in glass trays. Ordinary cell and fiber stains give satisfactory results. The method is particularly useful for certain neurophysiological purposes such as defining the location of electrode tracks and lesions and certain types of retrogrades. The Prussian blue test for electrolytically deposited iron can be conveniently applied in conjunction with other stains, to determine the point at which a given action potential response was observed, if steel electrodes are used.

The determination of the extent of ablations or of the locus of electrode tracks and electrolytic lesions in the brain is a problem encountered in many studies of central nervous functions. For most of these purposes serial sections are either necessary or desirable. Three years use of the frozen section method has led to a procedure which is adequate for many purposes and which can be conducted with considerable economy of time.

The chief obstacle to cutting serial sections of cerebral tissue by the freezing method is the formation of hard and brittle ice crystals which fracture the sections as they are cut. If the tissue is first treated with a 20–30% solution of ethyl alcohol for about 24 hours this difficulty can be avoided. The time required depends on the size of the block; enough time must be allowed to permit uniform penetration of the

alcohol into the tissue. It does not appear to matter how the alcohol solution is used. Any fixation solution to which alcohol can be added may be made up with sufficient alcohol to make a 20-30% solution. Thus the brain can be sectioned, without washing, 12-48 hours after perfusion of the brain. The alcohol can be also added after fixation has begun or is complete; or, after fixation, the brain may be washed overnight and then put into 20-30% alcohol in water for 24 hours and sectioned. In any case, the material to be sectioned should be cut into blocks approximately one centimeter thick. It is sometimes desirable to strip off the pia and usually necessary to remove the extensive vascular tissue at the base of the brain. This can be done most safely the second day of fixation.

When ready for cutting, the tissue is placed on a CO₂ ice freezing block, which has been covered by a piece of wet blotting paper. The solid CO₂ ice is ground in a mortar to a conglomerate of small pieces of ice and snow and the ice chamber is filled with the mixture. For large blocks a little 95% alcohol can be put into the ice chamber with the CO₂ ice to hasten the initial freezing, but the speed of freezing must be increased with caution. Any open places where definite structural discontinuities exist are filled with water. A narrow ice bank must be similarly built up around the edges of thick blocks. It is usually necessary to build up the bank and open spaces by adding water drop by drop, as the block freezes, over a period of 10-15 minutes. As soon as the block is frozen thruout, most of the CO₂ ice is removed from the chamber.

While formation of hard and brittle ice crystals is chiefly controlled by the low concentration alcohol solution, some precautions are necessary in cutting the sections. It is necessary to avoid freezing the block too quickly and to avoid freezing it any harder than necessary. With each stroke of the knife the cut section is removed by means of a camel's hair brush kept wet with 50% alcohol, and it is necessary to paint any ice banks around the block and the colder portions of the block with 50% alcohol. This can be done in a second or two. The appearance of the surface, the force required to push the knife, and the sound the cutting produces are all guides to the estimation of the proper degree of hardness. A little experience teaches the correct judging of this part of the procedure. The essential thing is to keep the surface of the block near the freezing point; the sections are cut from a surface frozen only hard enough to cut cleanly.

A Bausch and Lomb clinical microtome with an 11 cm. paraffin knife is used in the writer's laboratory.¹ The angle of the knife with the block is not critical; it may vary

¹Credit is due to Mr. J. I. Wexlin of the Bausch and Lomb Company for securing certain modifications of the frozen section microtome and for much helpful advice.

from 20° to 30°. The knife is usually set in a line perpendicular to the direction of motion. Tissue that has been fixed with acetic acid is cut by a razor blade clamped in a device which, in turn, is clamped in the regular knife clamps. There can be no objection to using this for any other type of work as we regularly cut with the knife fixed at right angles to the direction of motion. A freezing block is employed that is somewhat larger than that furnished with the microtome, but of the same general design. This block was cut out of a copper bar, but aluminum is probably to be preferred. There is no reason why other microtomes cannot be used, but the Bausch and Lomb model can be easily altered to give displacements of over 75 μ .

It is practical to mount sections 25–150 μ thick cut thru entire transverse frontal planes, or entire saggital planes of a cat's brain. There does not appear to be any reason why similar sections of larger brains cannot be cut with a correctly designed freezing block, but there has not yet been occasion to do so. As the sections are cut they are placed in order in an enameled pan or glass tray containing water or 30–50% alcohol and left there until the entire block is cut. Shrinkage and consequent curling of the sections can be kept at a minimum only by keeping them in a low concentration of alcohol or in water until they are mounted on the slides. It is helpful to lay glass strips in the pan which mark off the pan into alleys and thus keep the sections in order, in rows. Then they are mounted by placing the section or sections selected for a given slide in a dish of 50% alcohol. They are then floated onto the slide which has been previously coated with Mayer's egg albumin. Each section is smoothed out with a camel's hair brush and the slides are placed on a paraffin mounting tray or in an oven and dried at 40°–50° C. for 15 minutes to an hour.² The sections may also be pressed onto the slides with blotting paper, and the drying omitted. In any case, it is desirable to move the sections thru 70% alcohol to 80% alcohol to set the albumin before washing in water. Sections mounted from 50% or 70% alcohol adhere better than those mounted from water. Alkaline solutions should be avoided at each stage in the process of fixing and staining. If an alkaline fixative is used, the block can be made slightly acid by adding a little acetic acid sometime before cutting or the alcohol solution in which the sections are placed can be neutralized by addition of acetic acid. Even the laboratory water (pH 8) will detach the sections if they are washed in it for more than an hour. It is to be noted that silver processes usually involve an alkaline reducer. We have found that hydroquinone formalin reducer as recommended by Bodian (1937) does not detach the sections. Amidol at an acid pH has not been tried; (see Davenport, *et al.* 1939).

Gelatin in 2% solution is a satisfactory adhesive if all subsequent procedures are carried out at room temperature.

²The author is indebted to Dr. H. W. Magoun for this and several other suggestions incorporated into the procedure.

The mounting is one of the least satisfactory processes in the method, for stray bits of choroid plexus and overlapping edges of cortical gyri are sometimes not seen before the section is stained.

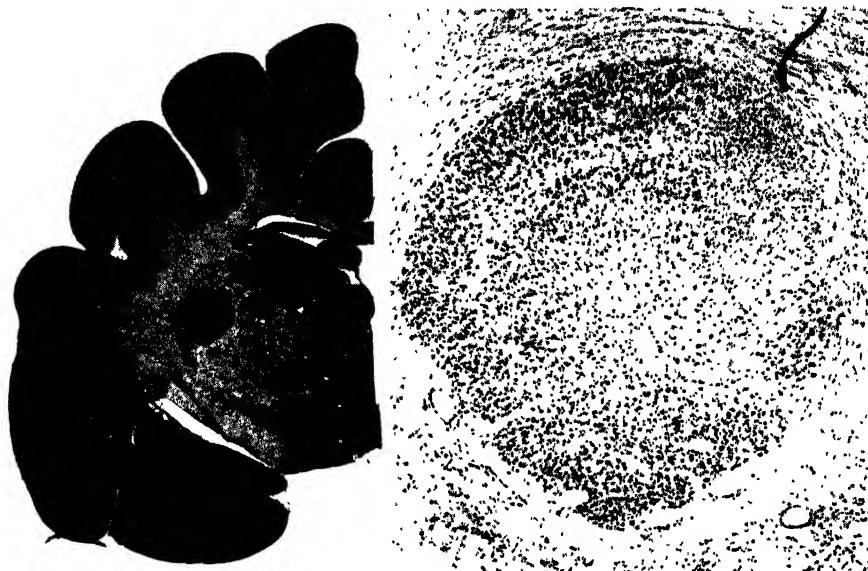
After the series has been mounted the slides are placed in glass trays and dehydrated thru a graded series of alcohol, placed in two changes of clove oil, ether-chloroform or xylene (preference in order named, but we usually use ether-chloroform) for approximately 15 minutes. After this treatment they are run down to water. Thionin (National Aniline and Chemical Company, Inc.), cresyl violet (suggested by Tress and Tress, 1935), toluidin blue (Coleman and Bell Company), and Weil's stains have given satisfactory results. For routine electrode placement sections, it is desirable to keep the differentiation time at a minimum. We have found that this can be done by washing the sections in distilled water 10-30 minutes after the above clearing process, then staining them 15-30 minutes at 50° C. in a saturated solution (less than 0.1%) made up in Sörenson's phosphate buffer calculated for a pH of 5.9. We concede that there is little justification for using this buffer because of the precipitating action of the phosphates. Critical illumination is of considerable aid in using these sections; see Carlson (1935).

There is no difficulty in cutting these sections serially with negligible loss thru a given block. We have not attempted to cut blocks thicker than 12 mm. This method is particularly well adapted for use in conjunction with a Horsley-Clark type of stereotaxic instrument, in which case the animal is perfused with fixatives to be described below, and the brain immediately blocked by partial cuts made by a sharp knife clamped to the needle carriage. These cuts overlap so that the brain is blocked in the plane of the electrode tracks. The animal is then removed from the stereotaxic instrument and the brain excised. When the brain is hardened the cuts are completed, making blocks the faces of which are approximately parallel with the electrode tracks, so that an entire needle track or a considerable portion of it is always seen in one section. For other purposes such as retrograde degeneration experiments it is desirable to outline the blocks similarly in the Horsley-Clark instrument, so that the nuclei desired can be included in a single block. It is, perhaps, worth noting that observation of the surface of blocks as they are cut affords a rather clear view of the gross orientation of electrode tracks.

Any of the usual fixatives should be satisfactory; those used by the writer are 10% formalin, 10% formalin in saline or Ringer's, 10% formalin in Ringer's with enough alcohol to bring its concentration up to 25%. The latter is necessary if sectioning is to be done the following day. It is possible that the fixation is better if the alcohol is not added to the perfusate, and the brain is kept in the formalin for two days, after which the alcohol can be added and the tissue cut a

day or two later. Fixatives causing appreciable swelling of the tissue are obviously undesirable for use in acute experiments in which an area of the calvarium has been removed. For this reason acetic acid fixatives are not entirely desirable for perfusion. We have occasionally used 20% formalin for the purpose of reducing swelling during perfusion with acid mixtures.

This is a very practical means of locating electrode placements, for the brain can be cut and necessary sections stained the day following the experiment.



A photographic reproduction of a transverse section ($\times 2.3$) thru the lateral geniculate nucleus of the cat and a photomicrograph of the lateral geniculate in a contiguous section ($\times 21.0$). The cat was perfused with the alcohol, formalin, Ringer's combination and the fixation was completed in same. The sections were cut at 75μ , and stained with 0.5% thionin (unbuffered water solution). The retrograde lesion, indicated by arrows, resulted from a small striate cortex lesion made 31 days before the animal was killed.

The Prussian blue test (Adrian and Moruzzi, 1939) for inorganic iron can be introduced in a very convenient way to locate with precision the point at which steel electrodes recorded a given action potential response. Immediately after the observations have been made a current of 10-20 microamperes is passed for 5-10 seconds thru the electrode by putting the positive side of a 3-4.5 volt battery to the electrode connection. The negative pole is connected to a diffuse ground. At the conclusion of the experiment the animal is perfused with a solution consisting of 10% formalin, and 1% potassium ferrocyanide, in Ringer's solution. After the brain is blocked as described above, the animal is removed from the Horsley-Clark in-

strument and the brain is excised and put into the same fixing solution plus enough acetic acid to make a 2% solution, and enough alcohol to make a 20% solution. A day or two later the brain can be sectioned without washing. The electrolytic deposits of iron are a well defined green, usually visible to the naked eye. Thionin in phosphate buffered solution used as described above provides an adequate stain for nuclear groups and contrasts well with the green of the iron; see Talbot (1940). It is also adequate for defining the extents of many types of experimental lesions (Bard, 1940), and is useful for many types of retrograde degeneration experiments. (Figs. 1 and 2.) It is to be noted that thicker sections prepared by this method contain no more tissue than ones 70% as thick cut from blocks imbedded in celloidin or paraffin because of the small amount of shrinkage. This factor is also of advantage in checking electrode spacings.

ACKNOWLEDGMENTS

The helpful advice and criticism from members of the departments of Physiology, Ophthalmology and Anatomy is gratefully acknowledged.

The author is also indebted to Miss Grace Futcher for technical assistance in developing this method.

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COMBINED FIXING, STAINING AND MOUNTING MEDIA

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ABSTRACT.—A number of non-volatile, water-soluble substances can be added to the usual aceto-carmine fixing fluids. These inert substances do not alter the fixation image and serve as mounting media when the volatile ingredients of the mixture evaporate. Formulae are given for solutions containing dextrin, dextrose, gelatin, pectin, sorbitol, and sucrose. Gum arabic can be incorporated in a formic-acid-carmine fixative. The limiting factor in the use of such mounting media in fixing fluids is the osmotic value they give the solution; with certain precautions, however, they can be used in place of the usual aceto-carmine treatment. The indices of refraction of these media are not as high as those of the natural balsams and the fixation images which the mixtures produce have the characteristic limitations of those secured by the aceto-carmine technic. Some of the natural balsams (Canada balsam, sandarac and Venetian turpentine) can also be incorporated in fixing fluids. These fixatives are able to hold balsam and water in solution together, and, as the volatile components of the mixtures evaporate, the fixed specimens remain in permanent balsam mounts. The addition of carmine to these fluids enables us to fix, stain, dehydrate, clear and mount a specimen in a single operation. These fixatives preserve more details of chromosome structure than the aceto-carmine fluids, but their use is more limited; and they can be substituted for the latter only with certain favorable material, e.g., pollen mother cells of *Rhoeo* and *Tradescantia* and salivary gland chromosomes of *Chironomus*. Some of the newer synthetic resins can be substituted for the natural balsams. Formulae are given for fixatives which contain Venetian turpentine, sandarac, Canada balsam and two synthetic resins.

Recently there has been a great increase in the use of cytological methods permitting microscopic examination of specimens immediately after fixation. Perhaps the most useful of these procedures has been to macerate or smear the specimens in 45% acetic acid saturated with carmine (Schneider, 1880), thus fixing, staining and mounting the material in a single operation. The addition of a trace of iron greatly intensifies the stain (Belling, 1921, 1926). This acetic-acid-carmine technic has proven especially valuable for investigation of meiotic divisions in pollen mother cells and for examining the giant

chromosomes in the salivary glands of the Diptera. The preparations thus made, however, are not permanent; and further treatment is necessary to preserve them for future reference (McClintock, 1929; Steere, 1931; Buck, 1935; Marshall, 1936; Bridges, 1937; Hillary, 1938, 1939; Burrell, 1939). The addition to the fixing and staining media of various inert substances, which do not alter the fixation image and which serve as mounting media when the more volatile components of the mixture evaporate, greatly facilitates the making of durable mounts. The very simplicity of such a technic gives it a number of practical applications.

These aceto-carmine fixing fluids which contain water-soluble mounting media can be applied successfully to a great many different types of material; in fact, if a few elementary precautions are observed, they can be substituted for the usual aceto-carmine mixtures. This method of making "permanent" mounts, however, has certain inherent limitations and should not be used when it is desired to preserve the finer details of chromosome structure. The water-soluble media have a lower index of refraction than the balsams and, while preparations kept in a dry place have shown no signs of deterioration over a three-year period, they are quickly destroyed if they come into contact with water. The most serious fault, however, is in the fixation image, for many of the finer details of the chromosomes are destroyed by 45% acetic acid. In fact, much of the more careful work of today avoids the acetic acid fixation image by means of a "pre-fixation" technic or by fixing first with a mixture which is less destructive than acetic acid, the aceto-carmine being retained in the technic primarily as a stain (Nebel 1939).

At present the balsams are by far the most satisfactory mounting media. Altho specimens have to be dehydrated and cleared before they can be impregnated with balsam, such preparations are used today almost exclusively. The studies herein reported, however, have shown it to be possible to make fixing fluids in which a balsam can be dissolved directly. The further addition of a dye produces a fluid which enables us to fix, stain, dehydrate, clear and mount the specimen in a single operation and thus simplifies greatly the making of permanent microscopic slides. As these fixatives preserve more detail than does 45% acetic-acid, and as the fixation images can be readily modified in a number of directions, preparations thus made have none of the disadvantages of those mounted in water-soluble substances. While this technic is not suited for preparing all types of material for cytological examination (it cannot be substituted generally for the aceto-carmine technic), it preserves specimens for which

it is fitted about as well as any of the more elaborate procedures.

Several of the newer synthetic resins can also be used as mounting media. They can be incorporated in fixing and staining solutions and treated precisely as the natural balsams. While none of those thus far investigated shows any particular advantages over the natural products, it is possible that some may be synthesized which will have optical properties and solubilities rendering them more satisfactory as mounting media.

I. WATER-SOLUBLE MOUNTING MEDIA

General Considerations. The more valuable water-soluble mounting media have been, with few exceptions, mixtures of two or more non-volatile substances. Farrant's solution (gum arabic, glycerin and water) and glycerin jelly (gelatin, glycerin and water) are typical of many such media. At least one component of each mixture must become an amorphous transparent solid on drying; and as these solids are generally too hard and brittle for permanent microscopic preparations, the practice has been to give them the proper consistency by adding to them a certain amount of glycerin. This has introduced the further complication, that when enough glycerin has been added, the fluids acquire such an osmotic value that they plasmolyze many of the more delicate specimens. Even when mixed with rapidly penetrating fixatives the glycerin may cause some shrinkage or prevent that swelling of the nuclear elements which has proved to be such a valuable attribute of many fixation images.

Indeed, the osmotic value of the non-volatile components limits very definitely the amount which can be incorporated in aceto-carmine fluids. On the other hand, these components must have a sufficient volume to occupy the space under the coverglass as the mount dries. Obviously, there will be a greater margin of safety if the substances which serve as mounting media have a relatively low osmotic value per unit volume. It is also necessary that the preparations dry out evenly and this demands that they be hygroscopic enough to pull water out from under the center of the coverglass to the edge where it can evaporate. For example, when gelatin is added to aceto-carmine, it solidifies very quickly around the periphery of the coverglass and seals off the rest of the mount from the air. The result is that the mount remains liquid for several weeks, and the subsequent uneven drying makes it worthless.

There are a number of solids which can be profitably incorporated in the aceto-carmine mixtures to form the basis of a mounting medium. Those investigated by the writer were dextrin, dextrose, sucrose, gelatin, gum arabic and pectin. These acquire a suitable

consistency, however, only when they are combined with some non-volatile water-soluble liquid. The liquids tested were glycerin, sorbitol (a hexa-basic alcohol) and gluconic acid. Glycerin was ultimately discarded in favor of sorbitol¹ for the latter has a larger molecule than glycerin and consequently has a lower osmotic value per unit volume. It also has a higher index of refraction and changes less under varying conditions of humidity. Gluconic acid apparently has no especial advantages.

It should be emphasized that the formulae here given are in no way definitive. They are merely samples of mixtures which have proved useful in mounting specimens of the pollen mother cells of *Rhoeo* and of several species of *Tradescantia*. They should be modified, of course, to suit whatever material is being investigated.

Dextrin. The dextrin should be c.p. and free from starch. The following formula is suitable for the pollen mother cells of *Tradescantia*:

| | |
|----------------------------------------------------------------|----------------------------|
| Dextrin | 10 g. |
| Sorbitol | 10 cc. |
| Acetic acid | 50 cc. |
| Water | 60 cc. |
| Fe(NO ₃) ₃ ·9H ₂ O | 0.5 g. |
| Carmine | to saturation ² |

The dextrin should first be dissolved in the water and the other components added in the order given. It is best to add the carmine after the ferric nitrate has been dissolved. The mixture should be brought to a boil and filtered.

Gelatin. If 10 g. of gelatin are mixed with 100 cc. of Belling's aceto-carmine (the gelatin should be dissolved in the water, the other components added and the whole boiled for 30 seconds and filtered), a liquid mounting medium is secured which automatically becomes sealed as the gelatin solidifies around the periphery of the coverglass. Such preparations dry out very slowly and may remain liquid for several weeks, the gelatin³ furnishing an excellent, if temporary, seal.

For more lasting preparations the gelatin must be combined with some substance which insures that the entire mount will harden slowly. In the past, glycerin has been used for this purpose, and

¹A satisfactory brand of sorbitol is sold by the Glyco Products Company, Inc., under the trade name of *Yumidol*.

²To saturate this and the following solutions with carmine, it is advisable to add 0.5 g. of the dye to each formula. This is somewhat in excess of the amount that will go into solution; but in the case of the venetian turpentine combination (page 147), it is a good safeguard to have some undissolved carmine at the bottom of the container.

³The gelatin is altered, of course, when it is boiled in acetic acid. It is not necessary for our purpose to identify the reactions.

glycerin may be incorporated in the aceto-carmine fluids. It is not recommended, however, for its osmotic value is too great in proportion to its volume. Parenthetically, it may be noted that the gelatin mounting media described below do not liquefy or soften in hot weather, and in this respect they are superior to the well-known glycerin-jelly. They have one defect, however, which need not be serious—small crystals are formed at the periphery. It should be emphasized that the exact proportions of the ingredients in these many-purpose fluids are to be modified to suit each specimen. The following formula may be useful as a point of departure for these necessary modifications:

| | |
|------------------------------------------------------------|---------------|
| Gelatin..... | 10 g. |
| Sorbitol..... | 10 cc. |
| Acetic acid..... | 50 cc. |
| Water..... | 60 cc. |
| $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ | 0.5 g. |
| Carmine..... | to saturation |

Ten grams of sucrose can be substituted for the sorbitol in the above, altho when this is done the mixture should be brought to a boil, but the excess carmine should not be filtered out. The undissolved carmine which remains insures that the solution will remain saturated, for otherwise the color slowly fades. If dextrose is mixed with gelatin, the mounting medium becomes too hard and brittle for practical uses. Gluconic acid and gelatin can be combined as follows:

| | |
|------------------------------------------------------------|---------------|
| Gelatin..... | 10 g. |
| Gluconic acid..... | 15 g. |
| Acetic acid..... | 45 cc. |
| Water..... | 55 cc. |
| $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ | 0.5 g. |
| Carmine..... | to saturation |

When gluconic acid is used, undissolved carmine should likewise remain in the solution. Even then the preparation may fade.

Gum Arabic, Gum Acacia. Gum arabic is soluble in 45% acetic acid but insoluble in stronger concentrations. Consequently, when it is added to aceto-carmine fixatives, the preparations become opaque when they dry in such a way that the water evaporates faster than the acid. On the other hand, the mount remains perfectly transparent if the acid evaporates faster than the water. Opaque mounts are avoided entirely if an acid more volatile than acetic is used. Pianese (1892) described a formic-acid-carmine combination which gives results in many ways more precise than the usual aceto-carmine fixatives, the chromatin being preserved with a sharper outline. As formic acid is also an aldehyde, it must never be mixed with gelatin.

The following is one of the most useful mixtures:

| | |
|------------------------------------------------------------|---------------|
| Gum arabic..... | 10 g. |
| Sorbitol..... | 10 cc. |
| Formic acid (87%)..... | 41 cc. |
| Water..... | 65 cc. |
| $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ | 0.5 g. |
| Carmine..... | to saturation |

If small bubbles appear around the periphery of the coverglass, the amount of sorbitol in the mixture should be increased slightly and the amount of gum arabic decreased. The gum arabic can be most easily incorporated in the mixture if first pulverized in a mortar and dissolved in the water.

Pectin. Pectin⁴ forms a gel in the presence of sugar and an acid. However, enough pectin cannot be added to aceto-carmine to serve as a mounting medium; other non-volatile substances must supply the volume. The advantage of using pectin lies in the fact that the preparation becomes solid as soon as it is made, altho it does not become really hard until it dries. There are a number of commercial brands of pectin available, the easiest to use being "Certo." Karo corn syrup (Patrick, 1936) is a satisfactory source of dextrose. The following mixture forms a usable mounting medium.

| | |
|------------------------------------------------------------|---------------|
| Certo (pectin)..... | 10 cc. |
| Karo (dextrose)..... | 10 cc. |
| Sorbitol..... | 5 cc. |
| Acetic acid..... | 55 cc. |
| Water..... | 55 cc. |
| $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ | 0.5 g. |
| Carmine..... | to saturation |

The mixture should be filtered and the excess carmine removed before the Certo is incorporated.

Directions. Aceto-carmine fixing fluids which contain mounting media are more viscous than those which do not. This introduces a complication in the smear technic, for delicate cells may be distorted in such a liquid by a too vigorous smearing. If pollen mother cells are to be fixed, the whole anther should be covered by the fluid for from 30 to 60 seconds before it is crushed. The cells should be examined immediately after they are extruded from the anther. They should not be shrunken; if they are, the mixture should be diluted with Belling's aceto-carmine. Very delicate cells can be more safely smeared in Belling's solution and then mounted in the

⁴For a detailed description of the pectin gels, see the contributions of L. W. Tarr, L. L. Baker and P. B. Myers in the Delaware Agr. Exp. Sta. Bull., 186-187. 1924-1934.

mixture. It is important, however, not to mistake the absence of the customary swelling of the cells for a shrinkage. This swelling, which is such a valuable attribute of the aceto-carmine technic, occurs only when the preparation is flamed. The mount should be heated almost to the boiling point to secure the optimum swelling and staining. The amount of solution to be used depends upon the size of the cover glass and the desired thickness of the finished preparation; three or four drops of the solution are generally sufficient. Flexible number 0 cover glasses adhere to the media better than do the thicker types. If some of the medium is extruded around the edge of the cover glass, it should not be removed for it greatly strengthens the mount. For the most satisfactory results, it is necessary to modify and adapt the technic for each type of specimen to be mounted.

II. FAT-SOLUBLE MOUNTING MEDIA

General considerations. To the best of the writer's knowledge, the technic of dissolving a balsam in a fixative has not been used hitherto. Under the circumstances it may be well to consider the principles involved in such a procedure in addition to the usual *ad hoc* directions and detailed recipes. Dissolving a balsam in a non-aqueous fixing fluid which gives an acceptable image offers no real difficulty. The only additional requirement is that the mixture be able to dissolve the water which is in the specimen itself without losing the balsam by precipitation. The addition of a reliable stain, however, introduces complications; in fact, hematoxylin and auramin were the only dyes found which stain the chromatin differentially when incorporated in these mixtures. Carmine^b was too insoluble to be used. The intensity of the hematoxylin and auramin staining, however, could not be controlled accurately, and consequently the definition was generally poor. Another disturbing factor, which tended to make the staining still more erratic, was the alteration of the staining properties of the solution on standing.

The addition of water to the fixing fluids resulted in two marked improvements: (1) it enabled them to dissolve carmine, a dye easy to control, and (2) it increased greatly the control of the fixation images. As far as chromosome structure is concerned, the image may be altered in any one of a number of directions by changing the proportions of the ingredients; in fact, almost any of the usual fixation

^bCarmine, of course, can be used in non-aqueous fixatives. In Carnoy's, for example, enough can be dissolved to stain the chromatin faintly. If methyl alcohol be substituted for the ethyl alcohol in the formula, a brilliant stain results. The fixation image, however, has little to recommend it and the mixture is not stable, the carmine being precipitated after a few days.

images are made available. On the other hand, the addition of water introduced another problem: it was necessary to hold the water and balsam together in a single solution, and this involved principles not generally met with in cytology.

Water and an oil can be made miscible by adding to them enough of a third liquid in which they are individually soluble. For example, if 10 cc. of xylene and 10 cc. of water are placed together in a graduate they will separate into two layers. If ethyl alcohol is added slowly, it will be partitioned between the xylene and water until 40 cc. has been added, whereupon it pulls the xylene and water into a single clear solution. In like manner it is necessary, if this technic is to prove practical, to hold both balsam and water in a single solution by reagents which, under these conditions, give a satisfactory fixation image.

The common reagents available in most laboratories of cytology differ greatly in their ability to hold water and an oil in solution simultaneously as is shown in the following table. The amounts are all expressed in round numbers, for the experiments were performed at laboratory temperatures and especially purified reagents were not employed.

TABLE 1. AMOUNT OF REAGENT REQUIRED TO HOLD WATER AND CERTAIN OILS IN SOLUTION SIMULTANEOUSLY

| | 10 cc. water and 10 cc. xylene | 10 cc. water and 10 cc. oleic acid | 10 cc. water and 10 cc. clove oil |
|------------------------------------------------------------|-----------------------------------|---------------------------------------|--------------------------------------|
| Methyl Alcohol | 90 cc. | 80 cc. | 70 cc. |
| Acetic Acid | 60 cc. | 115 cc. | 80 cc. |
| Dioxan | 60 cc. | 50 cc. | 25 cc. |
| Acetone | 50 cc. | 40 cc. | 30 cc. |
| Phenol | 50 cc. | 35 cc. | 25 cc. |
| Ethyl Alcohol | 40 cc. | 20 cc. | 20 cc. |
| Tert.-Butyl Alcohol | 30 cc. | 30 cc. | 25 cc. |
| Propionic Acid | 30 cc. | 40 cc. | 20 cc. |
| Ethyl Alcohol —2 pts. n-Butyl Alcohol—1 pt. } | 30 cc. | 25 cc. | 20 cc. |
| Phenol —1 pt. Acetic Acid —1 pt. } | 30 cc. | 20 cc. | 15 cc. |
| Phenol —11 pts. Propionic Acid—7 " Acetic Acid — 3 " | 21 cc. | 15 cc. | 19 cc. |

The reagents which are more effective in holding xylene and water in solution together are in general more effective in holding oleic acid and clove oil in solution with water. The table reveals, however, some marked exceptions. It is important to note that certain mixtures of the reagents are much more effective than any of the reagents used singly. For example, 60 cc. of acetic acid or 50 cc. of phenol are required to hold the xylene and water in solution, but when they are mixed in equal proportions only 30 cc. of the combination are needed. The mixture of phenol, propionic acid and acetic acid is the most effective yet found.⁶ This mixture can be used as the basis of a fixing fluid.

Fat-soluble mounting media differ greatly in their tolerance for water. Thus, gum mastic and gum damar proved so intolerant that they could not be incorporated in any practical fixative. On the other hand, Canada balsam, sandarac, and Venetian turpentine were employed successfully; but inasmuch as Venetian turpentine provides a much greater margin of safety than the other two media, it alone is recommended. Formulae containing Canada balsam and sandarac are included only for completeness.

Venetian Turpentine. The proportion of the several ingredients in the following formula is determined by three considerations: (1) The Venetian turpentine and water must be held in a clear solution which does not become cloudy on drying; (2) a sufficient quantity of carmine must be soluble for staining; and (3) the fixation image must be acceptable.

| | |
|------------------------------------------------------------|---------------|
| Venetian turpentine..... | 20 cc. |
| Phenol..... | 55 cc. |
| Propionic acid..... | 35 cc. |
| Acetic acid (glacial)..... | 10 or 15 cc. |
| Water..... | 25 cc. |
| $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ | 0.5 g. |
| Carmine..... | to saturation |

To this it is sometimes necessary to add 5 drops of ethylenediamine. This formula can be put together in a graduated cylinder as follows: The propionic acid is first poured in and then the viscous Venetian turpentine is thoroly mixed with it. Next is added the phenol; either as loose crystals or a liquid. [If liquid phenol (88%)

⁶When phenol, propionic acid and acetic acid are added slowly to the xylene and water in the proportions indicated, the mixture of the five liquids emulsifies readily. When barely enough of the reagents has been added to pull the xylene and water into a single layer, the solution is clear in transmitted light, but opalescent in reflected light, indicating that some of the molecules are grouped in colloidal particles. The possibility is suggested that the molecules are definitely oriented with regard to each other and that the fluid possesses a certain structure.

is used, due allowance must be made for the water it contains.] The acetic acid comes next, and then the water. The ferric nitrate should be dissolved before any carmine is added. Under no circumstances should the mixture be heated. It may be filtered after 12 hours, altho some undissolved carmine in the bottom of the container does no harm. Special care should be taken not to allow the fluid to come into contact with the skin.

The proportions of the several ingredients in this formula have been derived empirically. They will doubtless have to be altered to suit the different specimens to which it is applied. Under the circumstances, it may be well to indicate briefly the role of each ingredient and to show the effects of changing its concentration. As the part played by Venetian turpentine is relatively passive, its concentration may be held constant and the variations limited to the other components.

The phenol ties the Venetian turpentine into the fluid, altho propionic acid may be substituted for much of it without causing any precipitation. A decrease in the amount of phenol decreases slightly the solubility of the carmine. The limiting factor in regard to phenol is the fixation image, as this is controlled by a very complex relationship between the phenol, the acids and water.

The propionic acid may be safely held constant. It has the same fixing properties as acetic acid, but it holds the fat-soluble components in solution better. It can take the place of some of the phenol, but nothing is gained by making the substitution.

It is perhaps more convenient to vary the acetic acid than any other component. This acid ties the water into the mixture. When the fluid is first made, 10 cc. of the acid is sufficient. On standing, however, the fluid alters somewhat and consequently a slight change occurs in its fixing properties. The original fixing properties can be restored by the addition of 5 cc. of acetic acid⁷. It may be noted parenthetically that the fixation image of most of the meiotic phases remains constant even if the proportions of the several ingredients are altered as much as 50%. The first meiotic metaphase, however, is very sensitive to such changes and it is possible, by keeping the phenol and water constant and varying the acetic acid, to secure a number of the more popular images. The proportions given here preserve the chromatic elements of the chromosomes of *Tradescantia* as spiralized chromonemata. This choice of fixation image is, of

⁷Even after standing six months the characteristic image of the fresh solution can be restored by adding another 5 cc. of acetic acid.

course, somewhat arbitrary, altho when this image is chosen, homologous chromosomes in different cells show less individual variation than they do when they are fixed as parallel threads, or, at the other extreme, as swollen, heavily staining bodies with a central row of vacuoles.

The water controls the amount of carmine which the solution dissolves. The valuable swelling of the nuclear elements characteristic of the aceto-carmine technic also depends upon the presence of water.

The amount of iron mordant to be incorporated must be accurately determined. The best results for routine use are obtained by using either 0.5 g. of ferric nitrate or 0.2 g. of ferric chloride. If less iron is used, the stain is not sufficiently intense; if more is used, the iron tends to "salt out" the water so that the mount may become opaque on standing. This separation of water from the other ingredients as the mount hardens can be prevented by adding ethylene-diamine, which, by forming a complex cation with the iron, prevents the "salting out" effect. Thus, when it is desired to increase the staining capacity of the fluid, more iron can be included if at the same time ethylene-diamine is added. The quantities of each will have to be determined by experiments. Too much ethylene-diamine decreases the staining capacity of the mixture; too little allows it to cloud.

Canada balsam and sandarac. The following formula has given results comparable to those of the Venetian turpentine mixture. It is less tolerant of water, however, and consequently has a more limited usefulness.

| | |
|------------------------------------------------------------|---------------|
| Canada balsam or sandarac..... | 10 g. |
| Oleic acid..... | 10 cc. |
| Phenol..... | 65 cc. |
| Propionic acid..... | 40 cc. |
| Acetic acid (glacial)..... | 15 cc. |
| Water..... | 20 cc. |
| $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ | 0.5 g. |
| Carmine..... | to saturation |

Directions and precautions. The limiting factor in the use of fixatives which contain a balsam in solution is the water which is in the specimen itself. If too much water is introduced, the preparation clouds on drying. The remedy is either to fix a smaller specimen or use more of the fixative. The amount of fixative also depends upon the thickness of the preparation and the size of the cover glass. In making smears of the anthers of *Tradescantia*, three drops are enough for a 22 X 40 mm. cover glass and four drops for one 24 X 50 mm.

The following method has been successful with pollen mother cells of this species. The anthers are placed uncrushed on a *dry* slide and covered by one drop of the fixative, which is allowed to penetrate for from 30-60 seconds. The anthers are then smeared and two more drops of the fixative added. After standing a few seconds, the preparation is ready for the cover glass. The preparation should *not* be flamed. The characteristic swelling occurs immediately on fixation, altho the stain becomes slightly more intense during the first 24 hours. This slide is permanent as soon as it dries. No special precautions are necessary in the dry air of a steam heated laboratory; the slide may simply be left to harden on a table. On a humid day in summer, however, or in the moist air of a seaside laboratory, the water evaporates relatively slowly, while the acids and phenol evaporate at their usual rate. If the water evaporates more slowly than the other volatile components, the mount becomes opaque. This can be prevented by drying the slide on a hot plate or in a desiccator. It can even be dried in a paraffin oven (60° C.) without injury.

Limitations. These fluids have been used successfully in making smear preparations of small root tips of *Zea Mays* and in preparing permanent mounts of salivary gland chromosomes of *Chironomus*. They are suited, of course, for fixing, staining and mounting the pollen mother cells of many different plants. For some material, however, they are quite unsuited; e.g., the pollen mother cells of *Oenothera* and *Gossypium*. Apparently any specimen which contains tannic acid, or any other compound which forms a precipitate with the iron mordant, cannot be treated successfully with this technic.

III. SYNTHETIC RESINS

Recent advances in the synthesis of new resins promise to provide mounting media superior to any now in use. Both water-soluble and fat-soluble resins are available, and it is possible that some of these, which the writer has not investigated, have just the properties which are needed. None of the water-soluble resins which were tested, however, proved stable in the concentration of acetic acid necessary for the aceto-carmine technic. On the other hand, two of the fat-soluble resins were stable in the mixture of phenol, propionic acid, and acetic acid, and as they were slightly more tolerant of water than Venetian turpentine, they might be preferred to the latter under certain conditions. This advantage, however, is slight, and as Venetian turpentine has a higher index of refraction and adheres better to glass, it should be used in preference to these where possible.

*Duraplex V-240.*⁸ The formula for the mixture containing this resin is as follows:

| | |
|-----------------------------------------------------------|---------------|
| Duraplex V-240..... | 30 cc. |
| Phenol..... | 60 cc. |
| Propionic acid..... | 40 cc. |
| Acetic acid..... | 15 cc. |
| Water..... | 30 cc. |
| Fe(NO ₃) ₃ ·9H ₂ O..... | 0.5 g. |
| Carmine..... | to saturation |

This is to be used precisely as the Venetian turpentine mixture.

NY-83-8. This synthetic resin is handled more easily as a 70% solution in methanol. It does not adhere to glass as well as Venetian turpentine. The following formula can be used:

| | |
|-----------------------------------------------------------|---------------|
| Ny-83-8 (70% in methyl alcohol)..... | 30 cc. |
| Phenol..... | 50 cc. |
| Propionic acid..... | 35 cc. |
| Acetic acid..... | 20 cc. |
| Water..... | 30 cc. |
| Fe(NO ₃) ₃ ·9H ₂ O..... | 0.5 g. |
| Carmine..... | to saturation |

Lucite. Lucite is the trade name of a plastic made by E. I. du Pont De Nemours & Co. It is soluble in the mixture of phenol, propionic acid, acetic acid and water; and as it has a high index of refraction and adheres well to glass, it forms an excellent mounting medium. Two of its characteristics, however, render it unsuited for this technic. Mixtures which contain as little as 8% Lucite are so viscous that unless great care is taken, the cells of the specimen will be distorted when they are smeared in it. A more serious defect appears as the mount hardens. Fine droplets of water form (often temporarily) and any cells in contact with them become badly shrunken. The related mounting medium, isobutyl methacrylate, is even less tolerant of water than Lucite itself.

All grades of Lucite are soluble in the fixing mixture. Soft crystals (H7500) with type "D" plasticiser are easiest to handle. While passable slides can be made with the following recipe, it is not recommended.

| | |
|-----------------------------------------------------------|---------------|
| Lucite..... | 10 g. |
| Clove oil..... | 10 cc. |
| Phenol..... | 65 cc. |
| Propionic acid..... | 45 cc. |
| Acetic acid..... | 20 cc. |
| Water..... | 20 cc. |
| Fe(NO ₃) ₃ ·9H ₂ O..... | 0.5 g. |
| Carmine..... | to saturation |

⁸This is the trade name of a resin produced by *The Resinous Products and Chemical Co., Inc.*, of Philadelphia. The other resin designated Ny-83-8 is also their product.

CONCLUSIONS

Two types of mounting media which can be incorporated in fixing and staining solutions have been described. Those which are water-soluble can be added to the usual aceto-carmine fluids (gum arabic in formic-acid-carmine) and can be used wherever the usual aceto-carmine methods are suitable. The limiting factor is the osmotic value which the media give the solutions, so that sometimes special precautions are necessary. The swelling which is such a valuable consequence of acetic acid fixatives can generally be secured by heating the preparations to the boiling point. Slides made by this method have been kept in a dry place for three years and have shown no signs of disintegration. The fixation image has the characteristic limitations of that given by the aceto-carmine fluids.

Fat-soluble mounting media (Canada balsam, sandarac, Venetian turpentine) can be incorporated in a fixing and staining solution composed of phenol, propionic acid, acetic acid, and water. The fixation image given by this solution can be controlled more precisely than that of the aceto-carmine fluids. The limiting factor in the use of these fixatives is the water in the specimen itself. Sometimes special precautions are necessary in drying the preparations, but once dry they are as permanent as the usual balsam mounts. This technic cannot be used with material which contains tannin or other substances which precipitate the iron mordant. These fluids have been successfully employed on the pollen mother cells of *Rhoeo* and several species of *Tradescantia* and on the salivary gland chromosomes of *Chironomus*. They are not suited for preparing the pollen mother cells of *Gossypium* or *Oenothera*.

Synthetic resins can be incorporated in the mixture of phenol, propionic acid and acetic acid. One of them, Duraplex V-240, has a greater tolerance for water than has Venetian turpentine, altho it does not adhere to glass as well. Some of the newer resins will probably form better mounting media than any of the natural products now in use.

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THE SELECTIVE STAINING OF RED BLOOD CELLS

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ABSTRACT.—A method for the selective staining of red blood cells is described. Material is fixed in 10% neutral formalin in .85% NaCl and imbedded in paraffin or celloidin. Sections 6–10 μ are stained 1–5 minutes in chromotrope 2R. Basophilic and the less strongly acidophilic elements are decolorized with 5% phosphotungstic acid in 95% ethyl alcohol. Red blood cells and other strongly acidophilic elements that may be present in the preparation retain the chromotrope 2R. A counterstain of methyl blue may be used for staining the decolorized basophilic elements. As a result, erythrocytes are stained red by the chromotrope 2R, and basophilic elements blue, by the methyl blue. Less strongly acidophilic elements, having little affinity for either primary or secondary dye, are colorless or gray.

In a previous paper¹ the writer described a staining method for the differentiation of the acidophilic elements of tissue into two groups. Sections, appropriately fixed, were stained with erythrosin or other substituted fluorescein dye and differentiated in phosphotungstic acid. Following immersion of the sections in absolute ethyl alcohol, all tissue elements were decolorized with the exception of those strongly acidophilic. This group included erythrocytes, eosinophil granules, certain secretion granules, alpha cells of the pituitary, keratinized epithelia and Russell's bodies. The decolorized basophilic elements, collagen, reticulum, cartilage, bone, mucin, beta cells of the pituitary and basophil granules, were counterstained with anilin blue or light green. Less strongly acidophilic elements, including smooth muscle, striated muscle and the ground substance of most epithelia were characterized by their slight affinity for either primary or secondary dyes.

Since the publication of this paper, the technic has been simplified and a dye, chromotrope 2R, has been found superior to the fluorescein dyes for this procedure. The present technic is especially designed for the selective staining of red blood cells. The application of chromotrope 2R for staining the other above mentioned strongly acidophilic elements is under further investigation at this time.

¹Crossmon, G. C. 1939. Separation of the acidophilic elements of the tissues into two groups. *Anat. Rec.*, 73, 163–70.

The procedure is as follows:

1. Fix in 10% neutral formalin in 0.85% NaCl for 24-48 hours. Commercial formalin may be neutralized by the addition of marble chips a few days prior to fixation. Other fixatives may be employed but are not recommended for the preservation of red blood cells. Following fixation, material is washed in several changes of 70% ethyl alcohol to remove excess formalin.

2. Dehydrate completely, imbed in paraffin or celloidin, cut and mount as usual. It is important that sections be thin; with increasing thickness, decolorization in step 5 becomes more difficult. Six to 10 μ is suggested.

3. Stain sections in chromotrope 2R.

| | |
|------------------------------------------------------------|---------|
| Chromotrope 2R (Grübler or National Aniline Co.) | 0.25 g. |
| Distilled water | 100 cc. |
| Glacial acetic acid | 1 cc. |

A suggested staining time is 1-5 minutes.

4. Rinse sections in 4 or 5 changes of distilled water. The last change of distilled water should show no trace of dye.

5. Transfer sections to 5% phosphotungstic acid in 95% ethyl alcohol. Agitate the slides occasionally while in this reagent. Preparations are left in phosphotungstic alcohol until as observed under the microscope all tissue elements are decolorized with the exception of red blood cells. The time varies with the thickness of the sections and the thoroness of the distilled water rinse in step 4. Basophilic elements are readily decolorized. Less strongly acidophilic elements, especially striated muscle, may require as long as 2-3 hours.

The specificity of the stain has been tested by leaving sections in phosphotungstic alcohol for a period of 36 hours without the slightest decolorization of red blood cells.

6. Transfer to methyl blue.

| | |
|----------------------------------------------|---------|
| Methyl blue (National Aniline Co.) | 0.5 g. |
| Distilled water | 100 cc. |
| Glacial acetic acid | 1 cc. |

A suggested staining time is 2-5 minutes.

7. Rinse in distilled water.

8. Transfer to 2% glacial acetic acid for approximately 15-60 seconds. Sections should be examined microscopically at this time. As a result of this differentiation, erythrocytes should be red, less strongly acidophilic elements colorless or gray, and basophilic elements blue.

9. Pass thru 2 or 3 changes of absolute ethyl alcohol followed by 3 changes of xylol. Mount in balsam.

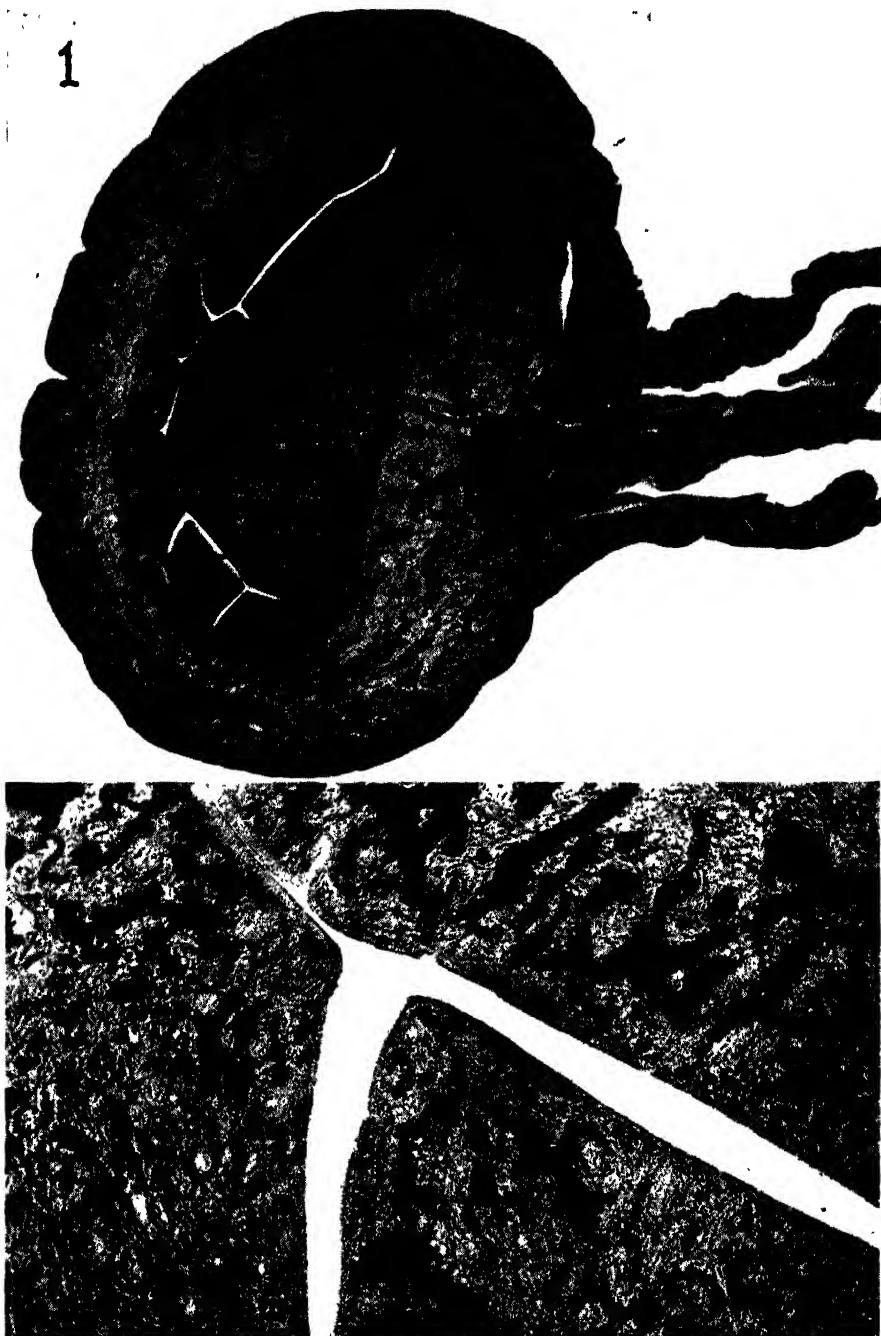


Figure 1. Photomicrograph of uterus of rabbit. Chromotrope 2R and methyl blue. $\times 10$.

Figure 2. Photomicrograph of the area indicated within the rectangle in Figure 1. $\times 100$.

RESULTS AND DISCUSSION

Staining results are as given in step 8. The method is selective for red blood cells but not specific. Other strongly acidophilic elements, if present, retain the chromotrope 2R. If it is desired, the technic may be simplified by omitting the counterstain of methyl blue. In this case erythrocytes are stained red on a colorless background of the less strongly acidophilic and basophilic elements. A nuclear stain of freshly prepared Weigert's iron hematoxylin may be used as a preliminary to staining with chromotrope 2R.

Altho thick sections are not suggested, the writer has decolorized celloidin sections up to 25 μ . Such preparations are very striking and are suggestive of carmine-injected material for the demonstration of the circulation.

ACID FUCHSIN AS A CONNECTIVE TISSUE STAIN AFTER PHOSPHOMOLYBDOTUNGSTIC MORDANTING

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ABSTRACT.—Certain acid fuchsins stain connective tissue deep red after phosphomolybdotungstic mordanting in a modified Masson procedure, others are entirely unsatisfactory for this purpose. Spectrophotometric examination gives no reliable criteria for separation of acid fuchsins satisfactory for this purpose from unsatisfactory ones. Sulphonation of basic fuchsin with 3.5 to 4 parts of 25–30% fuming H_2SO_4 to 1 part of dye gives a satisfactory product at temperatures as low as 65 to 70° C. in 30 minutes, while use of 5 to 7.5 parts of acid at this and at higher and lower temperatures gives unsatisfactory products. Satisfactory products may be produced with 15% fuming H_2SO_4 in similar quantities, and even with concentrated H_2SO_4 , but some unconverted basic fuchsin remains with both and, with the latter, lower quantities give unsatisfactory products. Brief chemical studies indicate that oversulphonation may occur in the manufacture of acid fuchsin and that this is just as deleterious as undersulphonation.

In the course of recent studies on trichrome staining (Lillie, 1938), it was noted that acid fuchsin (C. I. No. 692, lot NR-10) repeatedly failed to give a red connective tissue fiber stain after phosphomolybdic acid mordanting in a modified Masson technic. In this, acid fuchsin appeared to differ from the other commonly used diamino- and triamino-triphenylmethane sulphonic acid dyes such as light green SF (C. I. No. 670), fast green FCF, methyl blue¹ (C. I. No. 706), and anilin blue (C. I. No. 707). This discrepancy was quite disturbing from a theoretical point of view. Another old sample from Eimer and Amend also consistently failed to stain connective tissue.

Later two fresh lots of acid fuchsin, NR-11 and CR-6, were tested as fiber stains as a matter of routine confirmation of the previous results. Both of these samples repeatedly gave excellent red connective tissue staining by the following technic: Sections were first mordanted 1 minute in saturated alcoholic picric acid, washed 3 minutes in running water, stained 6 minutes in Weigert's acid iron chloride hematoxylin, rinsed in water, stained 4 minutes in 1% solution of naphthol green B (C. I. No. 5) in 1% acetic acid, rinsed in water, mordanted 1 minute in equal parts of 5% solutions of phos-

¹See Lillie (1940).

phomolybdic and phosphotungstic acids, passed directly into a 2.5% solution in 2.5% acetic acid of the acid fuchsin being tested and stained 4 minutes, differentiated 1 minute in 1% aqueous acetic acid, passed thru alcohol, acetone, acetone and xylene aa, and xylene into salicylic acid balsam.

All four samples gave satisfactory connective tissue staining when mixed with picric acid by the van Gieson method.

The possibility was considered that a predominance of rosanilin or pararosanilin sulphonic acids might be responsible. To rule this out, 10 g. lots of an old Grübler sample of rosanilin sulphate, and of a 1936-7 sample of new fuchsin from Hartman Leddon Company, were sulphonated with 50 g. 25% fuming H_2SO_4 , holding for 1 hour at 80-85° C. Both of the synthesized dyes gave good connective tissue staining.

Next the experiments of Scanlan, French and Holmes (1927) were repeated in part to ascertain whether the sulphonation conditions necessary to produce an acid fuchsin suitable for collagen staining after phosphomolybdtungstic mordanting are the same as those found best by them for acid fuchsins for van Gieson staining.

Our sulphonation technic is given here:

The fuming H_2SO_4 is weighed into a 150 cc. wide mouthed flask of general spherical form with flat bottom. The flask is clamped by its neck over a water or paraffin bath and a mechanical stirrer rigged in it. The bath is preheated to the desired lower temperature limit. Ten grams of finely ground basic fuchsin are then added gradually to the acid with the mechanical stirrer operating, avoiding boiling by slow addition of the dye. Then the sulphonation flask is immersed in the preheated bath and stirring continued for the designated length of time. When sulphonation is complete, a drop gives a clear red solution in water, which is decolorized by adding NaOH. Then the acid solution is poured into 350 cc. of cold water in a 2 liter beaker, and the flask is rinsed with 3 successive 50 cc. rinses of cold water into the same beaker. The diluted acid solution is then heated to boiling and neutralized with an amount of $CaCO_3$, slightly exceeding the quantity theoretically required by the amount of fuming H_2SO_4 used. For example, 40 g. of fuming sulphuric acid (30% free SO_3) requires approximately 43.6 g. $CaCO_3$; we used 45 g. The $CaCO_3$ is added gradually with constant stirring; the hot thick mixture is immediately poured into a large Buchner funnel and filtered into a vacuum flask. The large beaker is rinsed with successive washings to make a total of 500 cc. of boiling water and the washings are poured thru the precipitate. To convert the calcium salt to the sodium salt of acid fuchsin, 14 g. sodium bicarbonate is added, the solution is heated to boiling, set aside to cool over night, and the precipitate of $CaCO_3$ is filtered out. The solution is neutralized with 12-18 cc. of approximately 6 N HCl, testing with congo red paper. The deep red dye solution is evaporated to dryness over a steam bath. The yield is about 20 g. when 10 g. fuchsin and 40 g. fuming H_2SO_4 are used.

Ten-gram samples of a single lot of basic fuchsin were sulphonated for 15 minutes after the indicated temperature was reached, using at

each temperature 40 and 75 g. quantities of fuming H_2SO_4 , containing about 25% free SO_3 . The temperature ranges were 50–55° C., 65–70°, 80–85°, 95–100°, 110–115°, and 125–130°. Acid fuchsins NR-10 and NR-11 were used as negative and positive staining controls. The lots sulphonated with 40 g. fuming H_2SO_4 for 15 minutes at temperatures of 95–100° C. or higher were quite good, as was that sulphonated at 50–55° C. with 75 g. acid. The larger quantity of acid gave worthless products at 80–85° or higher temperatures, while with the 40 g. quantity of acid the lots prepared at the lower temperatures were all fairly good.

Following these tests, 10 g. lots of another batch of basic fuchsin were sulphonated with 40 and 50 g. quantities of 30% fuming H_2SO_4 at 65–70° C., holding for 15 and 30 minutes and one and two hours after the indicated temperatures were reached, making a total of 8 lots. Acid fuchsins NR-11 and E & A were used as positive and negative controls. This series indicated that the use of 50 g. of 30% fuming H_2SO_4 gave an inferior product and that with longer holding time, the product was poorer. With 40 g. acid the product was excellent and the optimum time appeared to be about 30 minutes. Consequently a larger batch was made with the same proportions, time and temperature. The product was excellent and colorimetrically apparently of higher dye content than any of the commercial samples.

To determine further the limitations on the proper quantities of basic fuchsin and acid for satisfactory sulphonation, another series of sulphonations was performed using the same temperature of 65°–70° C. and 30 minute time interval as previously.

In this experiment 12 g. portions of basic fuchsin were combined respectively with 40, 35, 30 and 25 g. portions of fuming H_2SO_4 containing 30% free SO_3 ; with 41.7, 36.2, 31.0 and 26.0 g. of a mixture of equal parts of the above fuming H_2SO_4 and of ordinary concentrated sulphuric acids; and with 42.5, 37.4, 32.0 and 26.7 g. of concentrated H_2SO_4 .

The processes using the mixture of sulphuric and fuming sulphuric acids, and concentrated sulphuric acid alone yielded, at the stage of treatment with sodium bicarbonate, color precipitates which were soluble in hot water and in HCl and which reprecipitated quantitatively with picric acid solution. These precipitates appear to be unaltered basic fuchsin.

The final products were tested as usual in the Masson technic. Satisfactory products were obtained from sulphonation with fuming sulphuric acid using 40, 35 and 25 g. quantities, from the mixture of fuming and concentrated sulphuric acids in all quantities tried, and

from concentrated sulphuric acid in 42.5 and 37.4 g. quantities, each to 10 g. of basic fuchsin. The products were unsatisfactory with 30 g. fuming H₂SO₄ and with 32 and 26.7 g. concentrated H₂SO₄. (While H₂SO₄ containing only 15% free SO₃ appears to yield satisfactory acid fuchsins, the yield is reduced and some fuchsin escapes sulphonation.)

TABLE I. COMPARISON OF VARIOUS ACID FUCHSINS IN THE VAN GIESON TECHNIC*

| Sample Designation | Amount of acid fuchsin per 100 cc. picric | | | | Rating by Masson Method |
|--------------------|-------------------------------------------|--------|---------|---------|-------------------------------|
| | 25 mg. | 50 mg. | 100 mg. | 200 mg. | |
| CR-6 | + | ++ | +++ | ++ | +++ |
| NR-11 | + | ++ | +++ | ++ | ++ |
| NR-10 | ± | + | ++ | + | - |
| E & A | - | ± | + | + | - |
| 75-125-1 | - | ✗ | ± | + | + |
| 75-110-1 | ✗ | ± | + | + | ✗ |
| 75-95-1 | - | ✗ | ± | + | ✗ |
| 75-80-1 | ± | ± | + | ++ | ✗ |
| 75-65-1 | ✗ | ± | ± - + | + | + |
| 75-50-1 | ± | + | ++ | ++ | + - ++ |
| 40-125-1 | ✗ | ± | + | + - ++ | + - ++ |
| 40-110-1 | ± | + | + | ++ | + - ++ |
| 40-95-1 | ± | + | ++ | ++ | + - ++ |
| 40-80-1 | ± | + | + | ++ | + |
| 40-65-1 | ± | ± - + | + | ++ | + |
| 40-50-1 | ± | ± | ± - + | + | + |
| 100/25-65-30 | + | ++ | +++ | ++ | +++ |

*The symbols in this table indicate:

+++ Excellent, ++ Very good, + Good, ± Fair, ✗ Poor, - No collagen staining.

As a measure of further control, many of these acid fuchsins were tested also by the van Gieson technic using various proportions of acid fuchsin as shown in Table 1. Fairly good correlation is shown but the van Gieson method does not give the sharp failures that become evident when the Masson method is applied.

Spectrophotometric examination of two samples CR-6 and NR-10, the one giving bright red connective tissue in the Masson technic, the other not, showed absorption maxima at 540 to 550 m μ in both.

TABLE 2. TRANSMISSION OF LIGHT BY ACID FUCHSINS CR-6 AND NR-10*

| Wave Length in m μ | 500 | 520 | 530 | 535 | 540 | 545 | 550 | 555 | 560 | 570 | 580 | 590 | 600 | 620 | 660 | 700 | % Trans-mission |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----------------|-----------------|
| CR-6 | 75 | 65 | 58 | 53 | 49 | 50 | 50 | 46 | 65 | | 95 | | 100 | 100 | 100 | 100 | % Trans-mission |
| NR-10 | 75 | 65 | 56 | 60 | 49 | 50 | 49 | 54 | 60 | 75 | 95 | 100 | 100 | 100 | 100 | % Trans-mission | |

*Note. Acknowledgment is made to Dr. M. I. Smith for these two examinations.

Believing that further spectrophotometric examinations might be of value, a number of spectrophotograms were prepared with the recording spectrophotograph of the Washington Biophysical Society and measured in detail. Table 3 gives a summary of these measurements. Characterizing NR-11, CR-6, 40-65-15, 40-65-30 and 100/25-65-30 as satisfactory acid fuchsins from the staining tests, and E & A, 75-125-1, 75-110-1 and 50-65-2 as unsatisfactory, no spectrophotometric character was found which would clearly separate these groups.

The comparatively high absorption percentage given by lot 50-65-2 (50 g. fuming H₂SO₄ to 10 g. fuchsin, sulphonated at 65-70° C. for 2 hours) at its absorption maximum when in aqueous solution at 5 γ per cc. indicates that this unsatisfactory sample has a comparatively high dye content. Hence it would seem that low dye content alone was not the reason for the poor staining. Doubling the acid fuchsin content of the 2.5% acetic acid solution used after the phosphomolybdate mordant, while it did slightly improve the connective tissue staining, did not render it satisfactory. The E & A sample, the 75-125-1 and the 50-65-2 lots were thus tested. Except for CR-6, all satisfactory samples show a ratio of the absorption percentage at 530 m μ to that at 560 of 1.1 to 1 or higher, but on direct comparison of the stains CR-6 is better than NR-11. The median of

TABLE 3. VARIOUS SPECTROPHOTOMETRIC CONSTANTS OF ACID FUCHSINS

| Sample | % Ab. 530 | % Ab. 520 | % Ab. at max, with 5γ per cc | λ max. Ab. % | λ 95% Ab. | Median λ 95% Ab. |
|-----------------|-----------|-----------|------------------------------------|-----------------|--------------|------------------------|
| | % Ab. 560 | % Ab. 570 | | | | |
| Satisfactory | | | | | | |
| NR-11 | 1.20 | 1.91 | 27.7 | 542-544 | 538-550 | 544 |
| CR-6a | .917 | 1.15 | 35.9 | 547-548 | 542-556 | 549 |
| CR-6b | .982 | 1.29 | 34.2 | 547-550 | 541-553 | 547 |
| 40-65-15 | 1.24 | 1.80 | 38.7 | 541-548 | 536-550 | 543 |
| 40-65-30 | 1.155 | 1.78 | 44.3 | 545 | 537-550 | 548.5 |
| 100 25-65-30 | 1.11 | 1.57 | 45.6 | 545-548 | 539-553 | 546 |
| Unsatisfactory | | | | | | |
| E & A a | .97 | 1.115 | 17.0 | 549-550 | 536-557 | 546.5 |
| E & A b | 1.00 | 1.07 | 27.7 at 10 γ | 548-549 | 531-559 | 545 |
| 75-110-1 | .98 | 1.37 | 17.2 at 20 γ | 545 | 544-550 | 547 |
| 75-125-1 | .923 | 1.06 | 18.2 at 20 γ | 549-550 | 544-553 | 548.5 |
| 50-65-2 | 1.017 | 1.54 | 44.0 | 547 | 540-553 | 546.5 |
| NR-10 (Visual) | 1.10 | 1.40 | About same as CR-6 | 545, 550 | | |

the zone showing over 95% of the maximum absorption percentage was between 545 and 549 with all the unsatisfactory samples, while the satisfactory samples ranged from 543 to 549, with 2 of the best at 546 and 548, the best and the poorest of the satisfactory samples at 543.5 and 544.

Chemical analyses of 5 samples were made by Senior Chemist Elias Elvove for nitrogen, total sulphur and free sulphate, sodium, calcium, magnesium and chlorine. After deducting calcium and magnesium, free sulphate and chlorine and enough sodium to satisfy the excess of anions over that required by the calcium and magnesium

and dividing the residues by the atomic weights, the following atomic proportions were obtained. (Table 4).

TABLE 4. ATOMIC PROPORTIONS OF N, S, AND NA IN ACID FUCHSINS

| Sample | CR-6 | 100/25-65-30 | 75-125-1 | 50-65-2 | E & A |
|--------|------|--------------|----------|---------|-------|
| N | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 |
| S | 3.86 | 2.52 | 5.80 | 3.54 | 1.50 |
| Na | 0.97 | 2.25 | 6.60 | 1.78 | 2.90 |

These results, while crude, indicate that the satisfactory samples 100/25-65-30 and CR-6 contain 2 to 3 sulphonic acid groups, that the marginal sample 50-65-2 probably contains 3 to 4 and that the 2 unsatisfactory samples 75-125-1 and E & A are markedly over-sulphonated and under-sulphonated respectively.

CONCLUSIONS

Acid fuchsins may be a satisfactory collagen stain after phosphomolybdic-phosphotungstic mordanting. If sulphonated with an excess of fuming H_2SO_4 or at too high a temperature, the product is unsatisfactory, probably on account of over-sulphonation. Too low temperature or insufficient amount and concentration of SO_3 also yield unsatisfactory products, probably on account of undersulphonation. The absorption spectra of satisfactory and unsatisfactory samples are similar.

ACKNOWLEDGMENTS

To Technician Coey E. Jones for aid in the sulphonations, staining of sections and ruling and measuring the spectrophotometograms.

To Mr. Allen of the Industrial Hygiene Division for taking the spectrophotometograms.

To the Washington Biophysical Society for the use of their recording spectrophotometograph.

To Senior Chemist Elias Elvove for chemical estimations.

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THE ACETO-CARMINE METHOD FOR FRUIT MATERIAL

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ABSTRACT.—It is not easy to make good aceto-carmine preparations of plants with small chromosomes at meiosis because the cytoplasm readily takes up the stain and this prevents a sharp differentiation. The staining reaction depends on the composition of the pre-fixative, the duration of fixation, strength of aceto-carmine and amount of iron used. These factors can be varied independently. Since not only species but their varieties differ markedly from one another in their behavior, the best results can be secured only after experiment with individual plants to discover the most suitable combination. Suitable combinations of these factors for some fruit plants are described. In general they demand (1) a weaker solution of aceto-carmine and more iron than has hitherto been used in the aceto-carmine technic, and (2) the introduction of iron and carmine into the pre-fixative. Iron acetate is added to a dilute solution of carmine in glacial acetic acid until the solution assumes a deep red color, without precipitation, and this solution is used as the acetic acid component of an acetic-alcohol pre-fixative. Anthers are colored purple by treatment with this fixative, but since it has only a mordanting effect they need to be smeared and stained in the ordinary way.

Plants with large chromosomes, such as *Tradescantia*, give excellent results with aceto-carmine; those with small chromosomes give inferior results owing to the readiness with which the cytoplasm takes up the stain and thus prevents adequate differentiation between chromosomes and cytoplasm.

Experience in this laboratory with the treatment of small chromosomes in various fruit species leads to the belief that what is needed is a radical modification of the fixation and mordanting methods used with aceto-carmine. With such modification these methods might be capable of giving as satisfactory results with small chromosomes as with large.

The staining reaction depends on four factors which can be varied independently: (1) constitution of the pre-fixative; (2) duration of fixation and storage; (3) strength of aceto-carmine solution; (4) amount of iron introduced.

Constitution of pre-fixative. Three parts of absolute alcohol to one

of glacial acetic acid is as a rule quite a satisfactory pre-fixative for aceto-carmine. Variation in the proportion of alcohol and acetic acid alters the quality of fixation but does not appreciably change the staining reaction. Poor fixation, however, results in a poor staining reaction.

Improved staining results can be obtained by introducing both carmine and iron into the fixative. Glacial acetic acid is boiled with excess of carmine and filtered after cooling. A saturated solution of iron acetate in glacial acetic acid is added drop by drop until the carmine-acetic solution assumes a deep red color, without precipitation. This carmine-iron-acetic solution is used to make the 3:1 alcohol-acetic pre-fixative. Occasionally an undesirable precipitate forms in this fixative, either at once or some time after the anthers have been put in. This occurs when the original carmine-acetic solution is too concentrated. The procedure to adopt is to test small quantities of the solution for precipitation after diluting with various amounts of glacial acetic acid.

Anthers placed in carmine-iron-acetic-alcohol become purplish in color, but since the fixative has only a mordanting effect they need to be smeared and stained in the ordinary way. When the chromosomes have been pre-mordanted in this way, they enjoy the fullest advantages of differential staining. The fixative is suitable for all fruit plants except those which do not require much iron, e.g. *Ribes* (see schedule).

Duration of fixation and storage. Very soon after fixation (2-3 hours) satisfactory chromosome staining can be obtained, but the cytoplasm has a granular appearance (Fig. 1). It becomes clear after fixation for 12-24 hours (Figs. 3-5). If the material is stored either in the fixative or in 70% alcohol for a considerable time (1-2 months) it is again difficult to obtain a preparation in which the cytoplasm is clear (Fig. 2). Long storage, however, produces a sharp differentiation between spindle and cytoplasm, a fact which is sometimes useful for studying abnormal spindle development or the positions of the chromosomes relative to the spindle.¹

To obtain a well-stained flattened cell it is essential to burst the pollen mother-cell wall without actually damaging the cell itself. In preparations made soon after fixation the cells are too delicate and are easily damaged, but on the other hand long fixation makes the cells too hard and it is not possible to burst the wall during the process of heating. The best time must be found by experiment.

¹Darlington, C. D. and Thomas, P. T. 1937. The breakdown of cell division in a *Festuca-Lolium* derivative. *Ann. Botany (London)*, n. s. 1, 747-61.

For most fruit plants, fixation for 12–24 hours gives the best results, but for *Ribes*, the cells are so delicate that they cannot be satisfactorily smeared for three days. Hardening can, however, be accelerated by transferring the material to Carnoy.

Strength of aceto-carmine solution and amount of iron introduced. La Cour² (1937) advocates dilution of the gentian violet stain for sharp and rapid differentiation of large-chromosome nuclei which take up the stain too readily, and it proves that by far the best root tip preparations of fruit plants also are obtained after dilution of the stain. Hitherto Belling's saturated solution of carmine in 45% acetic acid has been generally advocated in staining schedules. But in the case of aceto-carmine, as in that of gentian violet, a diluted solution makes for easier differentiation in the pollen mother-cells of fruit plants. Generally a one-third strength solution (diluted with 45% acetic acid) is suitable, but the best concentration varies for different plants. Altho iron has been added to the pre-fixative, it is still necessary to add considerably more than usual at the time of smearing. The amount needed again differs markedly for different plants. Pears and apples, for example, require a weak carmine solution and a large amount of iron; *Ribes* requires a weak carmine solution with only a trace of iron. The strength of the aceto-carmine and the amount of iron required are also dependent on the time of storage, weaker amounts being necessary after long storage.

The iron should be introduced during the process of smearing by teasing with iron needles or small scalpels, since aceto-carmine to which much iron has previously been added soon precipitates. The cytoplasm is less liable to become stained, however, if the time between smearing and heating is short; and with plants which demand a considerable amount of iron, it is necessary to dip the needle once or twice into a solution of iron acetate.

It is important to realize that one species or even variety may differ markedly from another in its reaction to aceto-carmine. Indeed, varietal differences may override any characteristic of the species and the importance of adapting the technic to the needs of the individual variety or clone must never be overlooked. In this respect the differences between diploids and triploids of the same species are very remarkable. My colleague, Mr. Raptopoulos, finds it impossible to obtain satisfactory staining for certain triploid cherries unless the acetic-alcohol pre-fixative described above is used, while for diploids and tetraploids the precaution is scarcely necessary.

²La Cour, L. 1937. Improvements in plant cytological technique. Botan. Rev., 5, 241–58.

Explanation of figures

All the photographs (except Fig. 4) were made from temporary preparations at a magnification of $\times 1000$ and enlarged to $\times 3000$ for reproduction. Except for Fig. 4 all the material was fixed in the mordant acetic-alcohol fixative described in this paper.

Fig. 1. *Prunus divaricata Moseri* ($2n = 16$). Bivalents and univalents. Stained in $\frac{1}{2}$ strength carmine 5 hr. after fixation. Cytoplasm granular.

Fig. 2. *Prunus divaricata Moseri*. Bivalents and univalents. Stained in $\frac{1}{4}$ strength carmine 3 weeks after fixation. Cytoplasm not well differentiated.

Fig. 3. *Prunus spinosa* ($2n = 32$). 16 bivalents. Stained in $\frac{1}{3}$ strength carmine 36 hr. after fixation.

Fig. 4. Duke cherry ($2n = 32$). Quadrivalents and bivalents. Stained in $\frac{1}{3}$ strength carmine 24 hr. after fixation.

Fig. 5. *Pyro-cydonia* hybrid ($2n = 34$). Bivalents and univalents. Stained in $\frac{1}{2}$ strength carmine 24 hr. after fixation.

Thanks are due to Dr. D. Eissler for the preparation from which Fig. 1 was made.



Critical studies should always be made on the temporary aceto-carmine slides, but permanent slides are useful for record purposes. All the photographs in the accompanying plate except Fig. 4 were made from temporary slides.

Experimental schedule. The following general schedule of aceto-carmine technic is suggested for small chromosomes:

- (1) Fix the material in 1:3 acetic-alcohol prefixative and determine the optimum time after fixation when the P.M.C. wall can be burst without damaging the cell.
- (2) By diluting Belling's saturated solution of carmine in 45% acetic acid (with 45% acetic acid) find the highest concentration which allows of clear cytoplasm.
- (3) Determine the amount of iron which the particular plant needs for optimum chromosome staining without causing the cytoplasm to darken.
- (4) If the amount of iron required is considerable, pre-fix in the mordant acetic-alcohol mixture described above.

NOTES ON TECHNIC

CHECKING AND ADJUSTING THE ALIGNMENT OF OBJECTIVES IN THE BINOCULAR DISSECTING MICROSCOPE.—This note concerns two methods found to be useful in keeping the binocular lens systems of this type of microscope in fair alignment; the first is for less refined work, the second is for more accurate work.

In the first method, the check is begun by swinging any one of the three sets of objectives (high, low or middle magnification) into position. Next, an index card is placed upon the dissecting stage. Using *one* eye at a time, the exact periphery of *each* field of vision is outlined by punching small holes into the card with a needle. No less than four holes should be made at the terminals of any two diameters which cross at right angles. When *binocular* vision is again used, the two fields as outlined by the two sets of holes in the card, should nearly coincide. If they do not, the binocular system is out of proper alignment, and the following adjustment is suggested: view the card thru the *left* ocular and see that a set of holes in the card exactly coincides with the periphery of that field; next, *without moving the card* (weight it down), remove the objective barrel and barely loosen the tiny screws which keep the *right* objective plate in position. Then, if the screws have been loosened just enough so that the *right* plate may be moved with slight fingering, and if binocular vision is resumed, this *right* plate may be moved so that the *right* field will finally agree with the *left* field, as denoted by the set of holes in the card. This process must be checked repeatedly by opening and closing the *right* eye and moving the objective plate until the two fields agree.

The second method, which is a more accurate one, involves a refined technic in which a cross-hair ocular or ocular eye piece is used. The method of checking the adjustment remains the same as above, or it may be modified by aligning the *point* of intersection in the cross-hair unit with a tiny *point* made on an immovable index card. In explanation, it may be noted that the diameters of the light diaphragms of the left and right ocular lenses might never be exactly the same. Thus the points of coincidence must of necessity be in the central axis of each lens system. This axis ought then to include the *point of intersection* of the cross-hair and the center of the microscopic field, as indicated by the meeting place of two diameters drawn at right angles upon the index card. In this better method the principle is the same as that suggested above. The *left* side of the binocu-

lar system, containing the cross-hair unit is aligned, the ocular cross upon card cross, with exact central agreement. Next, the unit is moved to the *right* ocular position and the screws attending the *right* objective plate are loosened. This *right* plate is moved so that the crosses *again* exactly agree. Finally, the screws are carefully tightened.—CHARLES O. HATHAWAY, College of William and Mary, Williamsburg, Virginia, and FREDERICK F. FERGUSON, College of William and Mary—V. P. I., Norfolk, Virginia.

RESINS FOR SEALING GLYCERIN MOUNTS [WITH A NOTE ON THE USE OF CLARITE (NEVILLITE V)].—Among materials used for sealing glycerin, glycerin-jelly or glychrogel mounts to make permanent microscopic slides, resins comprise one group usually available in the biological laboratory. To use them, rings of diameter equal to that of the circular cover-slips selected are painted on microscope slides with a fine (No. 1-No. 3) camel's hair brush dipped in the resin; use of a turn-table insures regular rings. Just enough of the glycerin medium to occupy the space in the ring when the cover-slip is placed is applied with a pipette and, if necessary, is warmed to facilitate spreading. The correct amount can be determined only by practice and varies a little with various batches of glycerin jelly and glychrogel. If the preparation is warmed, it should be cooled first, otherwise sealed carefully with resin thinned with xylol or toluol. A second coat should be applied the following day.

In resins employed as seals, clearness and whiteness have been considered desiderata by the writers. Bell's cement, a proprietary seal, fulfills these requirements, but is expensive. Recently a great number of water-white resins have been synthesized of which a few have been tested by biologists as mounting media in place of Canada balsam and gum damar. Three of these are: Clarite (formerly called Nevillite V), developed by the Neville Co. of Pittsburgh, Penna.; Teglac 161, produced by Bakelite, Ltd. of London; and Distrene 80, by Honeywill and Stein, Ltd. of London. The writers have used Clarite (Nevillite V) both as a mounting medium and as a seal. Where great haste is not important, glycerin mounts are made using a store of previously "ringed" slides, and the seal is made using a solution of 60 parts of Clarite to 40 parts of toluol by weight. Where mounts need to be used within a few hours of preparation, the authors use "Cellobalm" made by dissolving 100 g. of ethyl cellulose in one liter of toluol with shaking at intervals over a day or two and then adding 200 g. of Canada balsam. The solution requires no filtration. A more viscous solution can be made by using smaller proportions of toluol.

With Clarite as a mounting medium some workers in our laboratory have found that the toluol solutions recommended by Groat¹ develop bubbles by contraction greater at one side than at others. It is noted, however, that this takes place when fresh slides are warmed for rapid drying. Where slides have dried at room temperature this difficulty is generally obviated. Kirkpatrick and Lendrum² in using the water-white Distrene 80 have added a plasticizer, tricresyl phosphate (7.5 cc. tricresyl phosphate, 40 cc. xylol, 10 g. Distrene 80) to prevent retraction and such a modification might be used with Clarite. The superiority of Clarite over Canada balsam and gum damar is most apparent in thick mounts such as whole mounts of large flukes (*Fasciola*), and *Amphioxus* where cells may reach 3 mm. in thickness.—J. L. MOHR and Wm. WEHRLE,³ University of California, Berkeley, Cal.

A NEW EMBEDDING SCHEDULE FOR INSECT CYTOLOGY.—Attention has recently been drawn to the fact that the carbolic acid and water technic simplifies the arduous task of sectioning yolk eggs. These difficulties have been experienced in a study of meiosis in the eggs of various sawfly species, and were successfully overcome by the use of *n*-butyl alcohol and phenol followed by the water treatment. Since much work is now being done on insect cytology and embryology, the time appears opportune to make the schedule available to others.

Petrunkewitch⁴ first pointed out that the inclusion of phenol in a fixative gives "a peculiar elastic texture to the tissues, unlike anything produced by commonly used fixing fluids." Slifer and King,⁵ however, found that sectioning after the use of one of Petrunkewitch's fixatives provides smooth, clean sections but that "Cyttoplasmic details were badly distorted and the chromosomes were almost unrecognizable." By a process of elimination they found that the use of a standard cytological fixative followed by 24 hours soaking in a 4% solution of phenol in 80% ethyl alcohol resulted in equally fine sectioning qualities and at the same time preserved the normal microscopical features. The process of dehydration and embedding was accomplished by means of ethyl alcohol, carbol-xylol and paraffin. After the paraffin had set, the material was blocked

¹Groat, R. A. Two new mounting media superior to Canada balsam and gum damar. *Anat. Rec.*, **74**, 1. 1939.

²Kirkpatrick, J., and Lendrum, A. C. A mounting medium for microscopical preparations giving good preservation of colour. *J. Path. & Bact.*, **49**, 592. 1939.

³Employed by the U. S. Works Progress Administration. Aid from this Administration (Official Project 65-108-113, Unit C-1) is hereby acknowledged.

⁴Petrunkewitch, A. 1933. New fixing fluids for general purposes. *Science*, **77**, 117-118.

⁵Slifer, E. H., and King, R. L. 1933. Grasshopper eggs and the paraffin method, *Science*, **78**, 366.

and the paraffin pared down until the end of the material was exposed and the whole was then soaked in water for 24 or more hours. Both the phenol and the water treatments are essential; the latter assists the paraffin in gliding over the microtome knife.

In certain species of sawfly the eggs are laid in pine and spruce needles which themselves are extremely tough and consequently add to the difficulty of sectioning the eggs. To prevent hardening during dehydration the *n*-butyl alcohol method of Zirkle was used but only with limited success. Distinctly better sections were obtained by means of the Slifer and King technic, but since the higher concentrations of ethyl alcohol and particularly the xylol have an inherent hardening effect, the following composite schedule was evolved: the results justify its recommendations.

| <i>Step</i> | <i>1</i> | <i>2</i> | <i>3</i> | <i>4</i> | <i>5</i> | <i>6</i> | <i>7</i> | <i>8</i> | <i>9</i> | <i>10</i> | <i>11</i> |
|-----------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|-----------|-----------|
| Water..... | 95 | 90 | 80 | 65 | 50 | 30 | 15 | 5 | 0 | 0 | 0 |
| Ethyl alcohol..... | 5 | 10 | 20 | 35 | 40 | 50 | 50 | 40 | 25 | 0 | 0 |
| <i>N</i> -butyl alcohol.... | 0 | 0 | 0 | 0 | 10 | 20 | 35 | 55 | 75 | 100 | 100 |
| Phenol..... | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 4 | 4 |
| Hours..... | ½ | ½ | ½ | 1 | 1 | 1 | 24 | 1 | 1 | 1 | — |

After step 10 the material and butyl-phenol was placed on an equal amount of solid paraffin, transferred to the oven and changed to pure paraffin after 16 hours or so. Infiltration should be completed in a further four hours. The embedded material was trimmed and soaked as recommended by Slifer and King. Adhesion of the sections was improved by using slides thinly smeared with albumen. The albumen film should be dried and the ribbon floated on with the aid of 35% ethyl alcohol.

The step at which dehydration is commenced varies with the fixative used, e.g., after 2BD, step 1; after Kahle's fluid, step 4; and after Kahle's without water, step 6. Fixation in Kahle's without water, which gives an excellent stain with Feulgen, requires only two hours, so that the whole fixing and embedding process can be completed in approximately two days.—STANLEY G. SMITH, Department of Genetics, McGill University, Montreal.

DIOXAN FOR BLEACHING.—To bleach material fixed in osmic mixtures, either with or without acetic acid, immerse it in dioxan. Bulk material may require up to three days; sections a much shorter time. No additional treatment is required.—J. J. ASANA, Gujarat College, Ahmedabad, India.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

CROOKS, D. M. The use of a translongitome in making and interpreting alternative transverse and longitudinal serial sections. *Science*, 91, 150. 1940.

Translongitome is the name given to a new microtome attachment developed for the purpose of obtaining transverse and longitudinal sections from the same block. Such a two-plane cutting attachment can be used in a rotary or a sliding microtome. The sections come off in the form of a continuous ribbon in which they have been cut alternately crosswise and longitudinally. The device fastens into the microtome clamp, and consists fundamentally of a hinged sector capable of swinging thru an angle of 90° in a plane parallel to the knife edge. It is, of course, necessary to set the microtome to cut one half the thickness desired as each face of the block is cut on alternate strokes. The translongitome is manufactured under patent rights, and priced at \$22.50.—*J. A. de Tomasi*.

ECKEL, EARL E. Cover slip dispenser. *J. Lab. & Clin. Med.*, 25, 882-3. 1940.

A machine is described which ejects cover slips one at a time in a position to be grasped by the edges without smearing the surfaces. The machine is composed of a plunger of correct thickness running on a horizontal plane thru a box containing cover slips, the edges of the box being raised sufficiently in the direction of the motion of the plunger to allow only one cover to be pushed out of the box. The box can be bent into shape from scraps of stainless steel or monel metal. The slit is adjustable for thickness of cover slip. The box and runway for the plunger is soldered to a heavy metal bar. The plunger is cut from the 0.006-inch blade of a thickness gauge. A pin is soldered to each end of the plunger and one to the back of the base to permit a rubber band to draw back the plunger.—*John T. Myers*.

JOHN, K. Ein neues Universalgerät. *Zts. wiss. Mikr.*, 56, 371. 1939.

A new universal microscope manufactured by Fuess of Berlin is described. It differs from the well known Leitz "Panphot" in placing the photographic portion of the apparatus to the left of the microscope on the same plane instead of superimposing the camera over the latter. An ingenious arrangement permits the light to be diverted either to the left for photomicrography or to the right for microprojection upon the drawing board. The illuminating unit and accessories are permanently built in and are suitable for opaque illumination as well as for work with the polariscope.—*J. M. Thuringer*.

MOSEBACH, GEORG. A microprocess for the cryoscopic investigation of succulent tissues. *Ber. deut. botan. Ges.*, 58, 29-40. 1940.

A microcryoscope is described by means of which osmotic concentrations may be determined on pieces of succulent tissue having a volume smaller than 1 cu. mm.—*Merritt N. Pope*.

PHOTOMICROGRAPHY

ARMITAGE, F. D. An eyepiece camera for miniature negatives. *The Microscope*, 4, 124-7. 1940.

The camera consists of two tubes and a focusing screen. The lower tube fits over a fiber washer that is placed over the eyepiece end of the body tube or draw-

tube, forming a light-tight joint. Between the two tubes of the camera are two diaphragms of fixed aperture; between these slides a metal plate serving as a shutter. At the top of the camera is a brass-bound wooden holder for the focusing screen or the double-sided plate-holder. The focusing screen is ruled in squares of $\frac{1}{2}$ cm. sides; in its center is a cover-glass cemented with Canada Balsam so that a focusing lens can be used. The negatives (4.5 x 6 cm.) will bear considerable enlargement.—C. E. Allen.

AUER, A. Die Verwendung des Rollfilms und die Messung der Belichtungszeit in der Mikrophotographie. *Zts. wiss. Mikr.*, 56, 259. 1939.

To determine exposure time for photomicrography the use of a small extinction type exposure meter such as the Zeiss Icon "Diaphot" is recommended. The aperture of this disc-shaped exposure meter is brought directly over the eye-piece. The gray wedge is rotated until the desirable microscopic detail just disappears. An exposure table is then made based upon known correct exposures and the corresponding meter reading for every lens combination used. For cameras with bellows, additional calculations must be made for various extensions.—J. M. Thuringer.

MICROTECHNIC IN GENERAL

BEYER, E. M. Trichrome stain for astrocytes. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 65-8. 1940.

A trichrome stain for astrocytes is described which gives results as good as those obtained with gold or silver impregnation and is suitable for bulk staining. It is better than Masson's trichrome stains for color photomicrography using Kodachrome or color separation. The method is as follows: Fix in formalin. Double embed (Beyer: Amer. J. Clin. Path., Tech. Suppl., 2, 173, 1938). Cut sections 5 μ . Remove paraffin. Run thru alcohols to water. Apply Muller's mordant, 8-12 hr. Wash in 3 changes of tap water and 1 of dist. water. Apply Hansen's hematoxylin, 5 min. or longer (Soln. A: ferric alum, amethyst cryst., 10 g.; ammonium sulfate, 1.4 g.; dist. water, 150 cc., heated below boiling until dissolved, and cooled. Soln. B: hematoxylin, 1.6 g.; dist. water, 75 cc., dissolved by heat, and cooled. A is added to B gradually with constant stirring, and when deep violet, as seen on filter paper, is heated slowly to boiling and boiled 90 sec., then cooled in ice water.) Wash in running tap water, 2 min. Differentiate in 2% acid alcohol, 15 sec. Wash in tap water. Rinse in ammonia water (1-2 drops to 100 cc.). Wash in dist. water. Stain in ponceau-fuchsin, 5 min. (Soln. A: ponceau de xylidine, either Eimer and Amend, Krall or Grubler, 1 g.; glacial acetic acid, 1 cc.; dist. water, 100 cc. Soln. B: acid fuchsin, Commission cert., 1 g.; glacial acetic acid, 1 cc.; dist. water, 100 cc. To 90 cc. of 0.2% acetic acid 6.6 cc. of soln. A and 3.8 cc. of soln. B are added). Wash in 2 changes of tap water and 1 of dist. water. Stain 5 min. in: phosphotungstic acid, 3 g.; orange G (Hollborn), 2 g.; dist. water, 100 cc. Mix in a mortar. Rinse quickly in 1 change of dist. water. Put in 0.5% Wasserblau (Grubler, sold by Pfaltz and Bauer) 10 to 15 min. Rinse in dist. water containing 1% of the Wasserblau staining solution. Run thru 2 changes of 95% alcohol, 2 changes of abs. alcohol, and then xylol. Mount in neutral balsam or gum damar.—G. H. Chapman.

CIARDI-DUPRÉ, G. Accorgimenti per allestire economicamente serie istologiche. *Monit. Zool. Ital.*, 50, 314-5. 1940.

An inexpensive substitute for slides and cover slips to mount large sections of embryonal and fetal tissue is described. Sections are mounted on old photographic glass which has been cut to the proper size and from which the gelatin has been peeled. Sheet cellophane, weighing 140-160 g. per sheet measuring 1 x 1.50 m. is used to cover the sections. Damar balsam is recommended as a mounting medium, and the cellophane, cut to appropriate size, is applied as one would a cover slip. It is important that the cellophane be under pressure for at least 24 hr. so as to obtain a level surface. The author suggests pressing between two thick pieces of cardboard held by a pair of elastic bands.—A. B. Dawson.

KARR, J. W. A substitute for balsam and damar. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 70. 1940.

Nevillite (Neville Company, Neville Island, Pittsburgh, Pa.) is suggested as a mounting medium.—*George H. Chapman*.

MC NAMARA, W. L., MURPHY, BERTA, and GORE, W. A. Method of simultaneous fixation and decalcification of bone. *J. Lab. & Clin. Med.*, 25, 874-5. 1940.

The following method of simultaneous fixation and decalcification can be used for the majority of special staining procedures: Cut sections of bone not more than 8-10 mm. thick with a scroll saw. Place them in the decalcifying solution at 37° C. Dissolve 10 g. of HgCl₂ in 300 cc. of dist. water with heat and cool it. Dissolve 90 g. of trichloroacetic acid in 100 cc. of water and add 5 cc. HNO₃, 40 cc. formalin and 50 cc. 95% alcohol. (Mix the two solutions.) Change this solution daily until tissue is soft. Keep in running water for 24 hr. (or in 2% NH₄OH for 12 hr. and running water for 24 hr.) Place in 80% alcohol, 2 hr.; 95% alcohol, 6 hr.; abs. alcohol, 16-24 hr.; xylol, 5-10 min.; cedar oil, 24 hr.; xylol (2 changes), 30 min.; 56°-58° paraffin (3 changes), 4-6 hr.; and embed. The average bone decalcifies in 3-5 days. If longer than 7 days is required, nuclear staining is impaired. By this technic tissue distortion is minimal and the staining capacity of the nuclei is undiminished.—*John T. Myers*.

O'BRIEN, H. C., and HANCE, R. T. A plastic coverglass, isobutyl methacrylate. *Science*, 91, 412. 1940.

Natural resins, like Canada balsam and gum damar, harden slowly and turn yellow in time. Nevillite, or Clarite, recently suggested by the General Biological Co., dries rapidly and is practically colorless. Isobutyl methacrylate, a plastic made by the du Pont Co. and costing about \$1.00 a lb., is similarly found to dissolve in benzene and xylene, giving a colorless solution. Moreover, it dries very hard within 5-10 min. and yields a preparation somewhat more brilliant than those mounted in Clarite. Its refraction index is 1.477, very near that of glass. Isobutyl methacrylate may also be used to replace the coverglass by simply dipping the slide into a thin solution of the plastic and draining off the excess. Immersion oil does not affect its surface, which can be wiped clean with alcohol and soft paper. The film produced is very thin, thus interference with light transmission is very small; but it will scratch. Scratches are removed by dipping the slide into the same plastic solution. Lastly, the film obtained can be written on with ordinary ink for purposes of labeling.—*J. A. de Tomasi*.

WARD, MARGARET C. Gum damar in place of thin celloidin for frozen sections. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 71. 1940.

Gum damar, used to affix frozen sections to slides, does not interfere with staining. The method described is as follows: Coat the slide with gum damar. Float section onto the slide and drain. Flush with 70% alcohol and blot with a blotter moistened with 70% alcohol. Stain as usual. Keep the slide horizontal while in xylol because it tends to loosen the section.—*G. H. Chapman*.

DYES AND THEIR BIOLOGICAL USES

FIGGE, F. H. J. Squid melanin: A naturally occurring reversibly oxidizable pigment. *Proc. Soc. Exp. Biol. & Med.*, 44, 293-4. 1940.

The ink of the squid (*Loligo pealii*) is a highly concentrated suspension of melanin, the particles of which are visible only by dark field illumination. Comparisons between this melanin and synthetic dopa melanin indicate marked reduction with sodium hydrosulphite and re-oxidation with potassium ferricyanide in both instances, whereas natural melanin exhibits more sluggish reactions. Squid melanin contains some substance absorbing about 10% of the light which is not reversibly oxidizable.—*M. S. Marshall*.

ORR, J. W. The histology of the rat's liver during the course of carcinogenesis by butter-yellow (*p*-dimethylaminoazobenzene). *J. Path. & Bact.*, 50, 393-408. 1940.

The dye "butter-yellow", obtained from British Drug Houses, Ltd. under the name "dimethyl yellow", was dissolved in oil and then added to the diet for the induction of visceral neoplasms.—*S. H. Hutner*.

ANIMAL MICROMECHANIC

BRUNSCHWIG, A., SCHMITZ, R. L., and JENNINGS, S. Selective localization of Evans blue (T1824) in subplacental portions of entoderm in the rat. *Proc. Soc. Exp. Biol. & Med.*, 44, 64-6. 1940.

The injection of pregnant white rats with 4 mg. of trypan blue was followed by localization as small blue granules in the columnar cells of the subplacental portions of the visceral entoderm. The experimental check followed observations of the localization of Evans blue (T1824), which accumulated in the macrophages and fibroblasts of the stroma around malignant but not benign tumors, following the injection accidentally of a pregnant rat.—*M. S. Marshall.*

DIGGS, L. W. and PETTIT, V. D. A comparison of methods used in the detection of the sickle-cell trait. *J. Lab. & Clin. Med.*, 25, 1106-11. 1940.

Five methods of detecting sickle cells were compared. The best one is the following moist stasis technic of Scriver and Waugh (Canad. Med. A. J., 23, 875, 1930): Place a rubber band around the proximal third of the finger and allow it to remain for 5 min. Puncture the distal end of the finger, seal a drop of the dark blood under a cover slip with vaseline and examine microscopically.—*John T. Myers.*

GOMORI, G. A method for staining of carious lesions in teeth. *Proc. Soc. Exp. Biol. & Med.*, 44, 250-8. 1940.

The following method permits rapid and accurate work, with human teeth or with large numbers of rat jaws: Fix in 80-95% alcohol or neutral formalin and wash in repeated changes of dist. water. Impregnate with 0.25-0.5% AgNO_3 for 12-24 hr. and wash for at least 24 hr. Reduce in 5% $\text{Na}_2\text{H}_2\text{PO}_4$ for 24 hr. and wash several hours in tap water. Fix in 2% $\text{Na}_2\text{S}_2\text{O}_3$ ("hypo") for 12 hr. and wash under the tap several hours. Stained jaws may be dehydrated in alcohol, cleared in cedar oil and examined directly under the dissecting microscope or they may be decalcified in 5-10% sulfosalicylic acid (not mineral acids) and embedded in celloidin. Carious areas are black; healthy areas are unstained.—*M. S. Marshall.*

IGNESTI, UGO. Avvertenze per la colorazione con la fucsina di Ziehl di materiale fissato in liquidi contenenti acidi. *Monit. Zool. Ital.*, 51, 80-2. 1940.

A modification of the Vannucci-Mallory method is proposed in which the acid fuchsin is replaced by Ziehl's fuchsin according to the method of Gallego. This modification permits differential staining following fixation with alcohol or fluids containing acid (Susa, Gilson-Carazzi, Zenker acetic, picro-sublimate, Sanfelice, and Bouin).

The essential modification is the omission of acetic acid from the solutions used in steps 1 and 3 of the following procedure: 1) Stain for 3-5 min. in dil. carbol fuchsin (5 g. Ziehl's fuchsin, dissolved in 10 cc. dist. water). 2) Wash in dist. water. 3) Differentiate for 5 min. in dil. formalin (formalin, 3 g., dissolved in dist. water, 10 cc.). For the rest of the steps, the original method is followed.

If nuclear staining is too intense, the solution in step 1 may be made with 2 g. Ziehl's fuchsin, and the time of staining reduced to 1-3 min.—*A. B. Dawson.*

JÄGER, R., and JÄGER, F. Fluoreszenzmikroskopie im auffallenden Licht unter besonderer Berücksichtigung der Struktur der Oberfläche der lebenden Haut und der Vereinfachung der Hilfsmittel. *Zts. wiss. Mikr.*, 56, 278. 1939.

This is a technical description of various methods employed in obtaining sharp surface contour pictures of the epidermis. The latter is rendered sensitive to ultra-violet light by washing the parts to be examined with a dil. solution of some fluochrome such as Primulin "O" (Grübler) or Auramine (Hollborn). The usual opaque illuminators are used.

The method should find various practical applications in studying the effects of soaps and cosmetics, industrial hazards, etc., upon the epidermis.—*J. M. Thuringer.*

JUGE, JEAN. *Les potentialités morphogénétiques des segments du membre dans la régénération du Triton (Autopode).* *Rev. Suisse de Zool.*, 47, 65-133. 1940.

A method for contrast staining of bone and cartilage in cleared specimens is described as follows: Fix 24-48 hr. in 4% formalin. Wash in running water several hours. Transfer to 70% alcohol for 24 hr. Remove the skin. Stain 1-3 hr. in acetic methyl green (methyl green, 3 g.; 70% alcohol, 100 cc.; acetic acid, 2 drops). Differentiate in several changes of 70% alcohol several hours until the desired color is reached; 95% alcohol, 2 hr.; abs. alcohol, 2 hr. Stain 12-24 hr. in alizarin (saturated solution of alizarin in abs. alcohol, 1 cc.; abs. alcohol, 100 cc.; acetic acid, 5 drops). Transfer to abs. alcohol for 2 hr. Clear in toluol or benzol for $\frac{1}{2}$ hr.

Material may be preserved in a mixture of 27 cc. methyl salicylate with 7 cc. isosaphrol, or in methyl salicylate alone. Cartilage appears green (but loses its color at the end of several months); bone is red.—*A. B. Dawson.*

KAUFMANN, W. *Occurrence of special cell groups at vascular pole of glomerulus in mammalian kidneys.* *Proc. Soc. Exp. Biol. & Med.*, 44, 227-30. 1940.

This is a report of a study of the juxtaglomerular corpuscles of Goormaghtigh, composed of agglomerations of cells. The author recommends fixation in Bouin's or Zenker's solution while fresh, paraffin embedding and sectioning serially, 4-6 μ . Hematoxylin-eosin fails to give adequate detail, Masson's trichrome stain is better and Mallory's phosphotungstic-acid-hematoxylin reveals good nuclear detail. Cytoplasmic vacuolization is visible by this method or with Mallory's anilin blue connective tissue stain. Intercellular fibrils are well brought out with Masson's stain or better with silver stains.—*M. S. Marshall.*

LANDAU, E. *Quelques réflexions sur les procédés d'imprégnation des neuro-fibrilles par le nitrate d'argent.* *Bull. d'Histol. Appl.*, 17, 65-80. 1940.

A method is described for the silver impregnation of paraffin sections from the nerve fibers of the central nervous system, as follows: Use material that has been fixed in formalin. Fasten the sections to slides with a very weak solution of gelatin, heating to an optimum temperature of 35° C. Remove paraffin and place in a 10% solution of neutral formalin 12-24 hr. or longer, protected from light. (The authors do not consider treatment with 95% alcohol desirable before impregnation.) After removal from formalin, rinse in dist. water, and transfer, in the dark, to a 20% solution of AgNO_3 . Hold in an oven at 35-40° C. for 1-2 hr. according to the thickness of the section. Rinse 1 sec. in dist. water and transfer to 20% ammoniacal silver. After 5 min. rinse briefly and reduce the silver by several drops of 1% neutral formalin. Repeat at intervals, controlling the reduction of the silver under the microscope.

The impregnation is continued until the desired result is obtained. The reduction may then be stopped by formalin, by a thoro washing in dist. water, or by the method proposed by Gros. After toning in AuCl_3 and fixing in $\text{Na}_2\text{S}_2\text{O}_3$, the sections may be differentiated in a $\frac{1}{2}\%$ aq. solution of potassium ferricyanide or 10-15% KI. When the differentiation is finished, the section is washed several hours in running water, dehydrated, and mounted either in Canada balsam or in the following fluid preferred by the authors: dist. water, 30 cc.; gum arabic, 30.0 cc.; refined sugar, 30 g.; glucose, 5 g.

This method has been used successfully for the cerebral cortex, the cerebellum and the bulb.—*Jean E. Conn.*

MC CARTER, JOHN C. *A silver carbonate method for oligodendrocytes and microglia for routine use.* *Amer. J. Path.*, 16, 233-5. 1940.

The following silver method is recommended for tissue of the central nervous system: Fix in 10% formalin. Treat frozen sections (20-25 μ) with a 1% solution of strong ammonia in dist. water for a few minutes, if the material is recently fixed, or over night if fixed for several weeks. Without washing, transfer to 4% aq. HBr (10% soln. of conc. hydrobromic acid) at 37° C. for 1 hr., wash in 2 changes dist. water and put into 5% aq. Na_2CO_3 . Add an equal volume of 5%

aq. ammonium alum (disregard ppt.) and leave the sections in this mixture for 1 hr. to 2-3 days. Wash in 2 changes dist. water and stain in Hortega's strong silver carbonate solution (5 cc. 10% AgNO₃, plus 20 cc. 5% Na₂CO₃, plus strong ammonia water to just dissolve ppt., filter and add 20 cc. dist. water) for 2-5 min. Reduce, without rinsing, in 1% formalin and agitate by blowing on the sections. Wash well in dist. water, gold-tone and fix in hypo in the usual manner. Dehydrate the sections after putting on the slide and cover in balsam. The method combines modifications of Hortega's method for oligodendrocytes and microglia with those of Globus and Penfield and adds the ammonium alum mordant; the time the sections remain in the first ammonia bath is varied to suit the time of fixation.—H. A. Davenport.

REXED, B., and WOHLFART, G. Über Färbung mit gepufferten Säure-fuchsinlösung. *Zts. wiss. Mikr.*, 56, 212-5. 1939.

The authors attribute the extreme variability of the action of acid fuchsin as used in Mallory's connective tissue stain to the varying pH of its solution. The reaction of a freshly prepared 0.1% solution of acid fuchsin is pH 4.49, and any alkalinity raises the pH sufficiently to render the stain inert. The following recommended formula gives a solution of pH 3.29±0.01: acid fuchsin, 1.0 g.; N/10 HCl, 60.0 ml.; dist. water, 900 ml.; Sörensen's citrate solution, 40 ml. (citric acid crystals 21.0 g., N/1 NaOH 200 ml., dist. water to make 1 L.).

Practically all organs stain in range of pH 3-4. The red blood cells alone stain at pH 5-7.—J. M. Thuringer.

RÖHLINGER, H., and REITZ, M. Die Methode der Schnittveraschung und ihre Bedeutung für die physikalisch-therapeutische Forschung. *Zts. wiss. Mikr.*, 56, 361. 1939.

The importance of the spodogram in dermatology for the control of physical therapy measures is discussed. Every functional change of the skin is contingent upon a shifting of its electrolytic components.

The presence of calcium, magnesium, phosphates, iron, and silicic acid are determined as follows: Fix the living tissue in abs. alcohol 48-72 hr. and imbed in paraffin. Cut sections 10 μ , mount with dist. water and incinerate with aid of microincinerator. Postmortem material is unsuitable because of its altered cell permeability, diffusion phenomena, and change in pH. The preparations are unstable and photomicrographs must be made immediately.

To determine total Ca salts: breathe gently on the spodogram to convert the water soluble Ca salts into carbonates which are practically insoluble. The residue may be washed from the preparation with dist. water.

The Mg pictures obtained after previous removal of Ca salts are less definite than when made from the fresh spodogram. It is demonstrated by treating the spodogram for 3 min. with a 1% tetraoxanthrachinon solution and removing the remainder with a 10% solution of antipyrin. Lastly, the sections are treated for 30-40 min. with dist. water (40-50° C.) using several changes.

The demonstration of phosphates is more difficult because of precipitate formation on the spodogram; however, they may be shown in the total ash as well as Ca picture. To overcome this difficulty a drop of abs. alcohol may be placed carefully on the spodogram and cover slip. The reagent ferric acetate (concentration not mentioned) is dropped at the side of the cover slip and drawn thru by holding a bit of filter paper to the opposite side. A few drops of alcohol mixed with the reagents hastens this process. This procedure should be repeated 3 or 4 times. A solution of 1% acetic acid in 39% alcohol is similarly drawn thru. Ferric phosphates are formed while other salts are removed by acetic acid. The iron salts are rendered visible by drawing thru first 2-3 drops of a potassium ferrocyanide solution followed by 4-5 drops of 1% HCl.

Iron may be demonstrated with the Berlin blue or still better the Turnbull reaction, as follows: precipitate with ammonium sulphide, add 20% potassium ferrocyanide in the presence of HCl and observe the blue color reaction.

Silicic acid is isolated by treating the spodogram ½-1 min. with 1-2% aq. HCl. The remainder of the spodogram consists of silicic acid since all other salts are dissolved and washed away by HCl.—J. M. Thuringer.

ROSKIN, G., and KIRPICHNIKOVA, E. Coloration vitale par ionophorèse. *Bull. d'Histo. Appl.*, 17, 146-7. 1940.

A method is described for vital staining of animal cells by means of ionization. Colored ions are introduced into the living organism by means of a continuous current from a storage battery having an intensity of 0.25 milliamperes for an electrode of 1 sq. cm. Tin plates are used for electrodes with the active one a little smaller than the other. Various dyes can be used; e.g., methylene blue which has a colored positive ion. The electrodes are attached to the animal after the hair has been removed. A pad of gauze soaked in a 0.5-1.0% solution of methylene blue is placed under the active electrode, and gauze wet with water is placed under the other electrode. The current is allowed to pass for 20-30 min.

Coloration can be observed after the first electrification, but it becomes more intense after this has been repeated on several successive days. With white mice coloring has been observed, not only in the cells of the skin, but also in the outer layers of the muscles. After killing the animal, the tissues are fixed in the following solution: Sat. aq. picric acid, 6 parts; sat. aq. ammonium molybdate, 2 parts; formalin, 2 parts. They are then passed thru alcohol and embedded in paraffin.

This method can also be used for vital staining of nerves.—*Jean E. Conn.*

SCHORR, E. A new technic for staining vaginal smears. *Science*, 91, 321. 1940.

The conventional hematoxylin-eosin-water-blue technic of Papanicolaou for vaginal smears is not entirely satisfactory when stress is put on the detection of cornification, a cytoplasmic change taken as a measure of ovarian function. The following modification of the Masson trichrome stain has, however, proved useful, because the sequence of contrasting colors produced is comparable to a chemical titration of the cell charge of the smear. Fix (no time given) the wet smear in 95% alcohol-ether, 1:1; run down to water. Stain 2 min. in Harris' hematoxylin; rinse 4-5 times in water, and let stand 5 min. in running water. Stain 5 min. in ponceau-acid-fuchsin-orange-G; rinse 3-4 times in water. Mordant 10 min. in 3% phosphotungstic acid; rinse 3-4 times in water. Counter-stain 8 min. in light green; do not wash. Acidify 3 min. in 0.25% acetic acid; do not wash. Dehydrate, clear, and mount. For the composition of the trichrome stain reference is made to Foot's description (*Amer. J. Path.*, 14, 245, 1938).—*J. A. de Tomasi.*

TAFT, A. E. A supplementary method for the study of Arachnopia. *Science*, 91, 272. 1940.

The usual preparations of brain and cord tissue give an inadequate idea of structure and histological relations of leptomeninges. Such stained preparations can, however, be supplemented with others which, prepared by the following simple technic, will often yield further valuable information: Float fragments of fresh or formalin-fixed tissue from water onto a glass slide, add a drop of glycerin, and put cover in place, with the application of some pressure. The material is thus made suitable for dark field work, a technic especially valuable when the study of vascular arrangement and meningeal concretions is called for.—*J. A. de Tomasi.*

TAKEYA-SIKO. Markscheidenfürbemethode am Gefrierschnitt. *Zts. gesam. Neurol. u. Psychiat.*, 169, 216-9. 1940.

As a supplement to Schroeder's article (abs. *Stain Techn.*, 15, 87) two methods for myelin sheaths are presented. The first is the Sugamo method as follows: Fix in 10% formalin; wash in running water 12-16 hr. Cut frozen sections (about 80 μ). Place 8-5 min. in 50% alcohol (agitate well); transfer to dist. water, then mordant 6-15 hr. at 37° C. in $K_2Cr_2O_7$, 5 g.; chromalum, 2 g.; dist. water, 100 cc. with sections flat and submerged in the fluid. Rinse 2-3 times in dist. water and stain 2-6 hr. at 37° C. in Kultschitsky's acetic hematoxylin. Transfer to water (use perforated spatula as sections are brittle); wash thoroly; then differentiate in 0.8-1.0% $KMnO_4$ 20-30 sec., one section at a time. Rinse in dist.

water and treat with a fresh mixture of equal parts of 1% oxalic acid and 1% Na₂SO₃. Wash thoroly in tap water; dehydrate; clear in creosote-xylene and mount in balsam. The Yatusiro method is like the first with the following exceptions: Omit the mordant; stain in aged Delafield's alum-hematoxylin 12-24 hr.; differentiate in borax 2 g., potassium ferricyanide 2.5 g., water 100 cc.—H. A. Davenport.

WOLF-HEIDEGGER, G. Die Anwendung von Kava-Kava bei der Fixierung des Dünndarms und anderer Hohlorgane. *Zts. wiss. Mikr.*, 56, 417. 1940.

Postmortem contractions of smooth muscle in the alimentary tract of mammals produce distorted histological pictures. To overcome this difficulty an infusion of powdered Kava-Kava root (*Piper latifolium*) is recommended.

The procedure is as follows: Heat 250 ml. Ringer's solution slowly to 80° C.; add 15 g. Kava-Kava powder, agitating constantly. When cooled to 37° C., take a few ml. of this solution and add a small amount of Merck's diastase, mix thoroly and add to the remainder of the Ringer's. Let digest in incubator for 2½ hr., filter, discard ppt., and proceed to inject animal. Any of the usual fixatives may follow this treatment.

The results were excellent on cats while rabbits receiving the same preliminary treatment did not respond to the Kava-Kava relaxation which may be attributed to their being vegetarians.—J. M. Thuringer.

PLANT MICROMECHANIC

GORDON, W. E. A labor-saving technique for leaf samples in histological work. *Science*, 91, 390. 1940.

For the purpose of recording the source of each leaf sample and maintaining its identity thruout the various manipulations in the laboratory, the following procedure is recommended: By means of a crow quill pen, put duplicate numbers in India ink on fresh leaves in the region from which the sample is to be taken. Punch out a disk-like portion which includes one of these numbers, and use this as a sample to embed and store for future sectioning. The other number is left to record the source and location of the sample taken; the leaf bearing it is pressed and dried for filing. As many as 15 such samples from the same specimen can be preserved, fixed, dehydrated and embedded at the same time with the sample bearing the number at the bottom of the block, so that it is legible thru the thin layer of paraffin. The numbers in ink are not washed off by chrom-acetic, or formol-acetic-alcohol fixatives, nor by the common alcohols, dioxan, or chloral hydrate.—J. A. de Tomasi.

HILLARY, B. B. Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction. *Botan. Gazette*, 101, 276-300. 1939.

"In vitro" experiments with nucleic acid impregnated agar blocks using four different types of fixatives showed that the Feulgen nucleal stain gives two types of staining curves, depending on the presence or absence of chromic acid in the fixative. With fixatives not containing chromic acid, the maximum stain is produced by hydrolysis at 60° C. extending from 4 to 8 min.; after that time a gradual falling off of the stain takes place, till at 30 min. no stain is visible. With fixatives containing chromic acid, the maximum stain is produced by hydrolysis at 60° C. from 5-30 min. That this greater retention of the stain with increased hydrolysis is due to chromic acid was established by testing the individual ingredients of the fixatives separately. Most of the ingredients of the fixatives, if they are present at the concentration normally used, do not interfere with the stain. Tannins, which occur widely in plant cells and which have been reported to inhibit the stain, were found to have no inhibitory effect unless a fixative was used that contained formalin. The nuclei of various plants from different divisions thruout the plant kingdom when treated in the same manner as the agar blocks showed the same type of staining curves. Causes of the previously reported negative stain reactions in many of these plants are discussed.—B. B. Hillary.

THOMAS, J. WARRICK. A comparison of cedar oil and other materials in the making of slides of atmospheric pollen. *J. Lab. & Clin. Med.*, 25, 1086-90. 1940.

Comparative studies were made of atmospheric pollen slides prepared with a glycerin jelly, glycerin vaseline, corn oil, almond oil and cedar oil. Glycerin jelly, containing a trace of methyl green (proportion not given) is desirable where structural detail is desired, but not for routine use. Cedar is preferable to other oils because it remains in the slide area to which it is applied, is more adhesive and makes the pollen grains more translucent. Cedar oil preparations may be filed either mounted with Canada balsam and cover slip, or unmounted in dust proof slide boxes.—John T. Myers.

MICROÖRGANISMS

HORVATH, J.v. Die Bedeutung des Zuchtwassers und der Fixierung in der Mikrotechnik der Protozoen. I. Die Wirkung des Eisennitrates bei Anwendung von Anilinfarben. *Zts. wiss. Mikr.*, 56, 291. 1939.

The staining of protozoa can be influenced by adding 5 drops of 0.18% $\text{Fe}_2(\text{NO}_3)_3$ to 10 ml. of the hay infusion or other culture medium in which they are growing. The action of the iron salt is said to be entirely unlike that of a mordant since it has no effect after fixation. It may be used in relatively high concentrations "in vivo" without affecting the viability of the organisms, and may be followed with one of the usual fixing fluids suitable for protozoa. The specificity of the various stains may be altered by regulation of the amount of $\text{Fe}_2(\text{NO}_3)_3$ added.—J. M. Thuringer.

LEVADITI, C., REINIÉ, L., STAMATIN, LE-VAN-SEN, and BEQUIGNON, R. Ultravirüs et fluorescence. Le virus vaccinal. *Ann. Inst. Pasteur*, 64, 359-414. 1940.

The following modified Hagemann technic is offered for the use of fluorescent dyes in the microscopy of viruses: Smear thick suspensions of elementary bodies on a slide, dry 1 hr. at 37° C. Agitate slides in dist. water for 10 min., dry 1 hr. at 37° C. Stain 5 min., rinse in dist. water, examine dry preparations by fluorescence. The authors used the Zeiss cardioid dark-field apparatus supplied with quartz stage accessories to enable use of an ultraviolet light source. The best dye, thioflavine, and the less suitable dyes, primuline, thioflavine S, auramine O, rhodamine B, trypaflavine, uranine A and eosin G, were obtained from Hoerlein. The elementary bodies are brilliantly fluorescent against the dark background.

Silver impregnation staining methods afford a good check on fluorescence methods.—S. H. Hutner.

HISTOCHEMISTRY

CRÄMER, G. Ein Verfahren, Nitrate im Gewebe sichtbar zu machen. *Zentbl. allg. Path.*, 74, 241-4. 1940.

The reagent, diphenyl-endo-anilo-dihydro-triazole (called "Nitron" by Busch), forms insoluble salts with nitrates and these can be demonstrated in tissue by examination with polarized light. The method is as follows: Dissolve 10 g. "Nitron" with 100 cc. of 5% acetic acid, heat (temp. not given). Cut fresh (unfixed) tissue with a freezing microtome (cool knife) and receive the section on a slide. Put 1-2 drops of hot "Nitron" solution on a cover glass and cover the section with it. Store $\frac{1}{2}$ hr. in a refrigerator to assist crystallization of the nitrate. Examination with polarized light reveals the presence of nitrates as doubly refractive zones. Methylene blue can be added to the "Nitron" reagent if desired. Sections must be examined immediately after removal from the refrigerator. A discussion of interfering substances is given. Further information: Busch, M., Ber. deut. chem. Ges., 38, 861.—H. A. Davenport.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the July number of this Journal.

STAINS CERTIFIED JUNE 1, TO AUG. 31, 1940*

| Name of dye | Certification No. of batch | Dye Content | Objects of tests made by Commission† | Date approved |
|------------------------------------|----------------------------|-------------|----------------------------------------------|---------------|
| Phloxine | CPh-3 | 90% | For histological staining | June 17, 1940 |
| Wright stain | CWr-13 | ... | As blood stain | June 27, 1940 |
| Rose Bengal | CRb-3 | 80% | As bacteriological stain | June 28, 1940 |
| Brilliant cresyl blue | NV-19 | 55% | For vital staining of blood | July 8, 1940 |
| Methyl orange | LM-1 | 91% | As histological counter-stain | July 16, 1940 |
| Methyl orange | NM-6 | 91% | As histological counter-stain | July 16, 1940 |
| Pyronin Y | NP-8 | 52% | As constituent of Pappenheim stain | July 22, 1940 |
| Methylene blue thiocyanate tablets | NAt-2 | 1.31-1.65% | For use in reduction test of milk | July 23, 1940 |
| Hematoxylin | FH-16 | | As histological and cytological stain | Aug. 2, 1940 |
| Fast green CF | LGf-1 | 98% | As histological and cytological counterstain | Aug. 10, 1940 |
| Malachite green | NMg-8 | 98% | As histological and bacteriological stain | Aug. 12, 1940 |
| Giemsa stain | NGe-6 | | As blood stain | Aug. 21, 1940 |
| Tetrachrome stain | NMn-11 | ... | As blood stain | Aug. 31, 1940 |

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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STAIN TECHNOLOGY

**GENERAL INDEX
VOLUMES 11-15**

1936-40

Published by the
BIOTECH PUBLICATIONS
GENEVA, N. Y., U. S. A.

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STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

VOLUME 16

1941

Published by
BIOTECH PUBLICATIONS
GENEVA, N. Y., U. S. A.

Printed in the U. S. A.

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STAIN TECHNOLOGY

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VOLUME 16

JANUARY, 1941

NUMBER 1

12279

ROMANOWSKY STAINING WITH BUFFERED SOLUTIONS, III. EXTENSION OF THE METHOD TO ROMANOWSKY STAINS IN GENERAL

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ABSTRACT.—Solutions at 0.3 g. per 100 cc. of equal parts of glycerin and methyl alcohol of various Wright, Giemsa, Leishman and Balch stains and similar eosinates of thiazene dyes give satisfactory wholesale staining of sections without differentiation when buffered with citric-acid and sodium-phosphate. Prestaining with alum hematoxylin adds to depth, density and permanence of nuclear staining, but decreases clarity. A satisfactory modification of Mayer's acid hemalum is described. The reaction should be pH 4.2 for neutral formalin or Orth fixation, pH 4.6 for acid formalin, pH 5.0 for Zenker formalin and pH 6.5 for ethyl or methyl alcohol or Carnoy fixation. Toluidine blue phloxinate is found to be a quite desirable stain and its preparation is described. Clarite and clarite X are definitely superior to neutral Canada balsam, and somewhat inferior in regard to fading compared with liquid petrolatum as mounting media for these Romanowsky stains.

Since the earlier publications (Lillie and Pasternack, 1932, 1936) this general technic has undergone some minor modifications which have tended to make it easier to use and to improve the keeping quality of the preparations. Recently a fairly extensive test of various commercial and experimental Romanowsky stains has been completed, which tends to widen the usefulness of the method. The purpose of this paper is to report these changes and experiments.

The basic purpose of the method is to adjust the reaction of the staining mixture so that no subsequent differentiation is required, and in fact should be avoided. This enables us to stain large numbers of sections simultaneously with consistent results, and to repeat the staining procedure precisely from day to day.

Apparently any of the compounds or mixtures of eosin and related

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dyes with thionin, toluidine blue, the azures and methylene blue may be used. The following were actually tried in a recent test, the results of which are presented in Table 1: Balch stain (lab. lot 1939), Wright's stain (CWr-12, NWr-10, and a pooled lot of surplus Army supply left over from 1918), Leishman's stain (Grübler), Giemsa stain (Grübler, Cummings, Biosol, Coleman and Bell 06-28-36, and NGe-3), thionin eosinate (Hartman Leddon 1938) and toluidine blue phloxinate (lab. lot 5-8-35).

The preparation of our laboratory Balch stain has already been reported in detail (1936). Toluidine blue phloxinate is prepared by precipitating toluidine blue with phloxine in aqueous solution in the approximate proportion of 2 mols (611.3) of toluidine blue to 1 mol (829.5) of phloxine B (C. I. No. 778) or 1 mol (758.5) of phloxine (C. I. No. 774). Since a slight excess of toluidine blue is desirable, 10 g. toluidine blue (62% dye content) in 500 cc. of distilled water is added to 10 g. phloxine B (82% dye content) in 500 cc. of distilled water. For phloxine, and for dyes of higher or lower dye content, the amounts are adjusted accordingly. After standing overnight, the precipitate is collected on hard filter paper with a suction filter and dried. The filtrate should be a very pale clear blue, indicating practical exhaustion of both dyes from the mixed solutions.

At first (1932) 1 g. eosinate of polychrome methylene blue to each 100 cc. of glycerin and methyl alcohol was used, but experience showed that there was a considerable residue of undissolved dye and the quantity was later (1936) reduced to 0.6 g. Further trial showed that 0.25 g. dye precipitate left no residue and 0.3 g. a slight residue. Comparison of the stain solutions thus prepared with the previous stock in which 0.6 g. had been used showed no appreciable difference in staining.

The Balch stain, the various Wright and dry Giemsa stain samples, the Leishman stain, the thionin eosinate and the toluidine blue phloxinate were dissolved at the rate of 0.3 g. in 50 cc. of C.P., neutral, anhydrous glycerin by standing overnight at 56°C. The glycerin solutions were then cooled and 50 cc. C.P. neutral methyl alcohol added. These constituted the stock solutions. The Giemsa stain solutions were used as furnished.

The buffer solutions are those previously described (1936) and consist of M/15 citric acid in 50% methyl alcohol and M/15 K₂HPO₄ or Na₂HPO₄ in distilled water. (See Table 2.) For the former sufficient accuracy may be attained by weighing out 14.01 g. mono-hydrated crystalline citric acid, dissolving it in 500 cc. distilled water and adding sufficient C. P. neutral methyl alcohol to bring

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TABLE 1. STAINING EFFECT OF 12 ROMANOWSKY STAIN SAMPLES WITH AND WITHOUT PRELIMINARY HEMATOXYLIN STAINING AT VARIOUS pH-LEVELS

| | pH | Wright CWr-12 | Wright NWr-10 | Wright war stock | Balch Lab 1939 | Leishman Grubler | Thionin eosinate | Toluidin blue phloxinate | Giemsa solution Biosol | Giemsa solution Gribbler | Giemsa solution *Cum-mings | Giemsa (dry) C & B 06-28-36 | Giemsa (dry) NGe-3 | |
|----------------------|-------------|---------------|---------------|------------------|----------------|------------------|------------------|--------------------------|------------------------|--------------------------|----------------------------|-----------------------------|--------------------|-----|
| Hematoxylin prestain | 4.2 | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | +++ | +++ |
| | 4.6 | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ± | +++ | ++ | ++ |
| | 5.0 | +++ | + | + | + | ± | + | + | + | + | ± | ++ | ± | ± |
| | No prestain | 4.2 | ++ | + | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 4.6 | +++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| | 5.0 | +++ | + | ++ | ++ | + | + | + | + | + | + | + | + | + |

++ = Excellent
++ = Very good

+ = Good
± = Fair

*This dye gave ++ staining with and without hematoxylin at pH 3.9.

the total volume up to 1,000 cc. after thoro mixing. For the phosphate solution, 9.47 g. C. P. anhydrous Na_2HPO_4 , or 11.62 g. C. P. anhydrous K_2HPO_4 is weighed out, using the specially purified salt designed for buffers. This is dissolved in distilled water. Recent experience in the south indicates that it would be well to use 25% methyl alcohol as the solvent for both buffer solutions. This prevents the deterioration due to the growth of molds.

TABLE 2. REACTIONS OF VARIOUS MIXTURES OF M/15 CITRIC ACID AND M/15 DISODIUM PHOSPHATE

| cc. M/15 citric acid | cc. M/15 Na_2HPO_4 | pH | cc. M/15 citric acid | cc. M/15 Na_2HPO_4 | pH |
|----------------------|------------------------------------|------------|----------------------|------------------------------------|-------------|
| 1.3 | 0.7 | 3.9(3.873) | 0.9 | 1.1 | 5.4(5.428) |
| 1.25 | 0.75 | 4.0(4.034) | 0.85 | 1.15 | 5.7(5.696) |
| 1.2 | 0.8 | 4.2(4.205) | 0.8 | 1.2 | 5.85(5.838) |
| 1.15 | 0.85 | 4.4(4.44) | 0.75 | 1.25 | 6.05(6.036) |
| 1.1 | 0.9 | 4.6(4.653) | 0.7 | 1.3 | 6.3(6.29) |
| 1.05 | 0.95 | 4.8(4.80) | 0.65 | 1.35 | 6.5(6.444) |
| 1.0 | 1.0 | 5.0(5.042) | 0.6 | 1.4 | 6.5(6.522) |
| 0.95 | 1.05 | 5.2(5.201) | 0.55 | 1.45 | 6.6(6.60) |

These figures are derived from Stitt's variation (1923) of McIlvaine's series.

When a fresh batch of buffer solutions is prepared, they are customarily checked by staining several sections at 0.2 pH variations in the expected range and selecting the proportion giving the best staining. This remains quite constant for the batch of buffer solutions.

The staining technic is as follows:

| | |
|---------------------------------------------------|--------|
| Stock stain solution as above..... | 2 cc. |
| Acetone, C. P..... | 3 cc. |
| Methyl alcohol, C. P..... | 3 cc. |
| Buffer solution to give the desired pH-value..... | 2 cc. |
| Distilled water..... | 30 cc. |
| | 40 cc. |

Bring paraffin sections to water as usual. Stain 1 hour in the foregoing mixture; rinse in water; dehydrate in acetone; pass thru a mixture of equal parts of acetone and xylene; clear in 2 changes of xylene.

Material fixed in formalin or Orth's fluid stains best at pH 4.2-4.6 (1.1 to 1.2 cc. M/15 citric acid and 0.8 to 0.9 cc. M/15 disodium phosphate); acid formalin material and slightly autolyzed autopsy material fixed in neutral formalin stain better at pH 4.6 or 4.8; sublimate formalin and Helly's or Maximow's formalin Zenker material are best at about pH 5.0 and material fixed in methyl alcohol, absolute alcohol or Carnoy at about pH 6.5. Decalcified material may stain better at 0.4 pH higher than similar undecalcified material.

The various stains used all give quite comparable and usually quite similar results. Toluidine blue phloxinate gives a particularly agreeable picture. The metachromasy of mast cell granules is perhaps more pronounced than with any of the polychrome methylene blue or azure eosinates, thus rendering them more easily distinguishable from somatic or bacterial chromatin. The deeper red staining of erythrocytes and eosinophil leucocyte granules is also quite desirable.

The proper pH-level for staining with any given batch of dye is best determined by staining a number of sections from the same block at 3 or 4 pH-levels varying by about 0.2 in the expected range for the fixation. This level will remain quite constant for the dye sample over a number of months at least.

Increased sharpness, density and permanence of nuclear staining may be attained by staining 3-5 minutes in an alum hematoxylin, washing thoroly and blueing in tap water (with 0.1 to 1% sodium acetate if necessary) before placing in the buffered Romanowsky stain mixture. Delafield's or Harris' formulae are quite satisfactory. A modified Mayer's acid hemalum, quickly prepared and quite stable, has been found very satisfactory. This is prepared as follows:

Dissolve 5 g. hematoxylin (certified) in 700 cc. distilled water, add 1 g. sodium iodate and 50 g. aluminum ammonium sulphate (ammonia alum). Dissolve. Let stand over night, then add 300 cc. C. P. neutral glycerin and 20 cc. glacial acetic acid. It is ready for immediate use.

Prestaining with hematoxylin, however, somewhat diminishes the clarity and brilliance of the stain. Sections prestained in hematoxylin may be mounted in neutral balsam; but without the hematoxylin and in daylight or under an electric light, sections fade fairly rapidly in neutral balsam as compared with sections mounted in heavy liquid petrolatum. The latter is undoubtedly the best preserving medium for this type of stain, but it is messy, it often leaks even with pyroxylin seals, and its index of refraction is rather low ($N_D = 1.483$). This low index of refraction is sometimes advantageous. Recently

trials have been made of clarite and clarite X as mounting media for these stains. So far the preparations have shown much less fading than have similar preparations mounted simultaneously in neutral Canada balsam and exposed to daylight or to an incandescent tungsten light under identical conditions. There has been slight fading as compared with sections mounted in the heavy mineral oil. No appreciable difference between these two new resins is yet evident after a month under continuous exposure to a 100 watt tungsten light at 20-25 cm. distance. The fact that these resins set hard is an obvious advantage over mineral oil.

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IMPROVED PARAFFIN SCHEDULES FOR PLANT TISSUES

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The two paraffin schedules here presented have produced less distortion in plant tissues than those commonly used. Both are modifications of the schedule described by Hemenway.¹ Schedule A is very similar to that described by Hewitt.² Schedule B usually causes less distortion than A but staining is not so bright as after A.

SCHEDULE A

- (1) Fix tissues 48 hours in Karpechenko, Flemming Strong (half strength)³ or other solutions that have been found most satisfactory for the tissues under investigation.
- (2) Wash tissues 6 hours in running water.
- (3) Place tissues on paraffin oven in an evaporating dish filled with the following solution: 100 cc. of 10% glycerin to which has been added 1 cc. of a 10% solution of thymol in 95% alcohol. (Put a canopy about 6 inches above the dish to allow air circulation but to prevent dust from accumulating in it.)
- (4) If tissues settle on the sides of the dish during evaporation of water, slide them toward the center of the dish so that they will remain immersed in the concentrating glycerin.
- (5) After all of the water has evaporated (10 to 13 days) transfer tissues to normal butyl alcohol and glycerin, 1:3, for 24 hours.
- (6) Normal butyl alcohol and glycerin, 1:1, 24 hours.
- (7) Normal butyl alcohol and glycerin, 3:1, 24 hours.
- (8) Pure normal butyl alcohol on oven, 24 hours.
- (9) Transfer tissues to new normal butyl alcohol in new vial and use new cork to eliminate film of glycerin present on first vial and cork. Leave on oven 24 hours.
- (10) New normal butyl alcohol on oven, 2 changes, 48 hours in each. (Agitate vial very gently twice daily while tissues are in butyl alcohol to hasten the removal of glycerin.)
- (11) Add new butyl alcohol and place solid blocks of paraffin on a

¹Hemenway, A. F. 1930. Some new methods and combinations in plant micro-technique. *Science*, **72**, 251-2.

²Hewitt, W. B. 1938. Leaf-scar infection in relation to the olive knot disease. *Hilgardia*, **12**, 41-65.

³Rawlins, T. E. 1933. *Phytopathological and Botanical Research Methods*. John Wiley and Sons, Inc., New York.

screen suspended in the upper portion of the butyl alcohol; place open vial in a well ventilated paraffin oven. Remove screen after 24 hours and allow remainder of butyl alcohol to evaporate in oven.

(12) Pour off melted paraffin and add new melted paraffin.

(13) Change melted paraffin 2 more times at 24 hour intervals and imbed after tissues have been in the last melted paraffin for 48 hours.

SCHEDULE B

(1) Follow schedule A thru step 10.

(2) Place tissues in normal butyl alcohol and cedar oil, 3:1, in an open vial and leave on oven until butyl alcohol has evaporated. (Use cedar oil labeled "for clearing".)

(3) Paraffin, 25%, in cedar oil, in oven, 24 hours.

(4) Paraffin, 50%, in cedar oil, in oven, 24 hours.

(5) Paraffin, 75%, in cedar oil, in oven, 24 hours.

(These mixtures of paraffin in cedar oil may be used a number of times before discarding.)

(6) Melted paraffin, 24 hours in oven.

(7) New melted paraffin, 24 hours in oven.

(8) New melted paraffin, 6 days in oven.

(9) Imbed.

A SECTION-SMEAR METHOD FOR PLANT CYTOLOGY¹

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ABSTRACT.—An improved method for making root tip preparations of plants with medium or small chromosomes is described. Division figures are oriented in polar view by cutting paraffin cross sections of roots; the pectic substance is removed from the middle lamellae and the sections then are smeared. The procedure recommended is as follows: Kill root tips in Navashin or other fluid containing chromic acid. Wash, dehydrate, embed in paraffin, and section transversely. Stain slides bearing the sections by a Feulgen technic following the modification of De Tomasi, except that the hydrolysis is prolonged to 45 minutes, and a 10-minute washing in running water is introduced between the fuchsin and the first bath in sulfite solution. Dehydrate and mount in thin Canada balsam; then apply local pressure to top of cover glass with the tip of a scalpel, while observing the action under low power of a microscope. The prolonged hydrolysis softens the tissue and removes sufficient pectic substance so that cells of the section separate readily and may be flattened until the chromosomes come to lie in a single plane. The slide is permanent.

Within the past few years root tip smear methods have proved themselves very useful in plant cytology, especially in those species with large chromosomes, such as *Tradescantia*, *Trillium*, *Vicia*, *Allium*, etc. Some difficulties have been experienced, however, in applying these methods to species with medium or small chromosomes. In these latter species, the chromosomes generally lie entirely within the metaphase plate, which is oriented at right angles to the long axis of the cell. When roots of these plants are smeared, the figures usually come to lie on the slide in equatorial view; and the small size of the cells makes it difficult to apply sufficient pressure to turn the figures so that they may be studied in polar view, without severely damaging them.

With the present method, which is largely a modification and combination of technics already in use, the difficulty is solved by applying pressure to paraffin cross sections of roots, and thus combining the proper orientation of sections with the flattening and spreading

¹This investigation has been supported in part by a grant from the Carnegie Corporation to the Carnegie Institution of Washington.

of smear methods. It was worked out on *Melandrium*, which has medium-sized chromosomes (2–6 μ in length) that are quite numerous in the polyploid forms.

The following is an outline of the section-smear schedule: Roots are killed and fixed 12–24 hours in Navashin or other suitable fluid containing chromic acid. They are washed, dehydrated and embedded in rubber-parowax,² using one of the newer dehydrating agents, such as *n*-butyl alcohol. When embedding, it is well to arrange roots from a plant in groups, all roots within a group being parallel and with tips even. Cross sections of roots (in the groups) are cut sufficiently thick to include whole division figures, but not so thick as to be two nuclei deep; 10 μ sections are about right for *Melandrium*. The sections of the embryonic regions of all of the roots in a group usually can be placed on a single slide. After drying the slides, the paraffin is removed in the usual manner and the sections are run down a graded series of alcohols to water. The slides are washed one-half hour or longer in running water (overnight washing does no harm and often removes the last traces of chromic acid stain from the tissues).

The Feulgen method³ is used for staining, except that slides should be hydrolyzed for 45 minutes instead of the shorter periods usually recommended. In addition, the slides should be washed in running tap water for 10 minutes upon removal from the leuco basic fuchsin and then passed thru three changes of sulfite solution, allowing 3 to 10 minutes in each change. After passing up an alcohol series to xylene, the slides are mounted in thin Canada balsam, using large, No. 1 cover glasses. The viscosity of the balsam is important in spreading the cells; a little experimenting will quickly show the proper dilution to use.

After the slides have partially dried (preferably 3 to 15 days after mounting) a figure with chromosomes well spread may be located, and while viewing this figure under the microscope (a 10 \times ocular and 20 \times objective are convenient), local pressure is applied to the top of the cover glass with the side of the tip of a scalpel. The scalpel is held in one hand, and the microscope is focused with the other, thus keeping the figure and the scalpel tip constantly in view. (A mechanical stage to hold the slide in a fixed position is almost a necessity.) The pressure serves to separate the cells and is best applied in the form of several firm strokes, just to one side of the section containing the division figure. The pectic substance of the middle

²Hance, R. T. 1938. A new paraffin embedding mixture. *Science*, **77**, 858.

³De Tomasi, J. A. 1936. Improving the technic of the Feulgen stain. *Stain Techn.*, **11**, 137–44.

lamellae largely dissolves out by the prolonged hydrolysis, and the repeated scalpel strokes should cause the cells to separate and float free in the balsam. Releasing the pressure rapidly at the end of a stroke often facilitates separation of the cells. Pressure should not be too great and should not be applied directly over a section, or the cover glass may break. With a little practice, the cell containing the desired division figure can be coaxed out undamaged from its original position in the section, with few, if any, adhering cells. One should not attempt to flatten the cell in its original position in the section; it must be isolated, comparatively free from other cells.

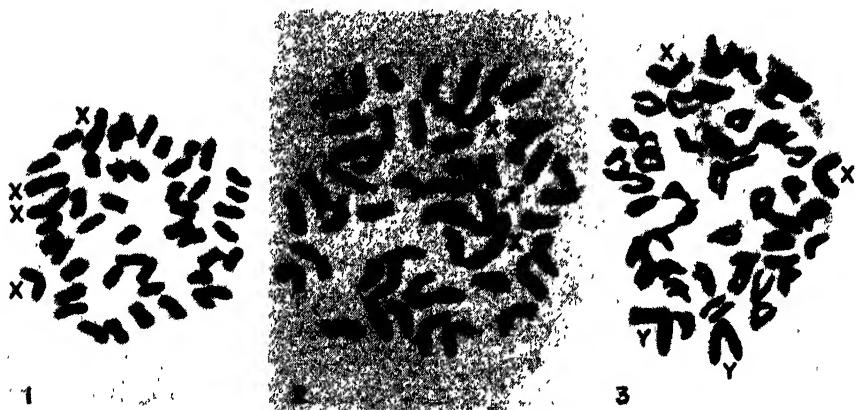


Fig. 1-3. Photomicrographs of section-smear preparations of *Melandrium* root tips, $\times 1400$: Fig. 1. Tetraploid female (4A + XXXX). Fig. 2. Tetraploid male (4A + XXXY). Fig. 3. Tetraploid male of the type 4A + XXXY. The chromosomes lie in one plane and are well separated. Note that sex chromosomes can be distinguished from autosomes, and that varying degrees of chromosome splitting and chromatid separation are shown.

Sufficient pressure is now applied (in the form of one or more strokes directly over the isolated cell) to flatten it and thus bring the chromosomes to lie in one plane (Fig. 1-3). The cytoplasm is soft after the hydrolysis, and gentle pressure is sufficient. If the pressure is released gradually after this flattening, the cell adheres to the slide and remains fixed in this position. The chromosomes now are ready for study. Or, if the first figure does not prove satisfactory, more figures may be located and the flattening process repeated. Figures may be studied before, during, and after flattening, and generally very little rearrangement or distortion of the chromosomes occurs. The preparations are permanent, being mounted in balsam, and will last almost indefinitely.

It is essential that a killing and fixing fluid be used which contains

chromic acid. Hillary⁴ has shown that hydrolysis may be lengthened to nearly an hour with no essential reduction in staining intensity after chromic acid fixation, but that 8 minutes' time is the maximum that can be employed without damage after fluids containing no chromic acid.

The 10-minute wash in running water immediately after the basic fuchsin is not included in the usual Feulgen schedule, and apparently is not necessary under some conditions. It greatly increases the intensity of the color reaction in *Melandrium*, however, and is essential to good, sharp contrast. When the basic fuchsin solution becomes colored because of repeated use, it may be regenerated by the addition of a few crystals of potassium metabisulphite ($K_2S_2O_5$).

The application of this method to *Melandrium* has enabled us to identify the sex chromosomes and to count accurately the autosomes in large numbers of plants.⁵ Figures 1-3 show the chromosome constitutions of tetraploid plants of three different types: XXXX female, XXXY male, and XXYY male, respectively. These figures show the chromosomes split and preparing for anaphase separation. In figure 2, the separation has occurred only in the region of the centromere, and was not visible until after the cell was flattened. In figure 3, the process has gone further; many of the chromatids are completely separate. A figure in this stage is usually unintelligible in ordinary paraffin sections, because the two separating chromatids lie in different planes. By this method, however, they are flattened and brought to lie side by side, where they can easily be studied.

The present technic is not recommended for root tip studies on species with large chromosomes; with species having medium or small chromosomes, however, it has proved to be more rapid, in the end, than the straight smear methods, because almost every slide provides a determination. It has been used almost exclusively on somatic tissue, but preliminary tests on sections of anthers and ovules indicate that it also may be useful in flattening and spreading meiotic division figures.

⁴Hillary, B. B. 1939. Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction. *Botan. Gazette*, 101, 276-300.

⁵Warmke, H. E., and Blakeslee, A. F. 1940. The establishment of a 4n dioecious race in *Melandrium*. *Amer. J. Bot.*, 27, 751-762.

STAINING BACTERIA AND YEASTS WITH ACID DYES

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ABSTRACT.—Various acid dyes prove satisfactory for the routine staining of bacteria. Those used are acid fuchsin, anilin blue w. s., fast acid blue R, fast green FCF, light green, orseiline BB, erythrosin, phloxine and rose bengal. Acid fuchsin, fast green, anilin blue, and orseiline are especially recommended. Phenolic solutions of the dyes, acidified with acetic acid, with the addition of ferric chloride to those containing acid fuchsin, anilin blue, fast green or light green, are used. Procedures are given in detail for staining or demonstrating vegetative cells, resting and germinating spores, capsules, sheaths and glycogen in bacteria; germinating and conjugating spores of yeast; and for counterstaining after acid fast or Gram staining. The principal advantages of using acid dyes are better differentiation, and less tendency for slime and debris to take the dye.

INTRODUCTION

Comparatively little use has been made of acid dyes for staining bacteria. Conn and Holmes (1926) state: "It had been assumed in the past that bacterial stains must be basic dyes; and the use of the acid dyes of this group (fluorescein) had never apparently occurred to bacteriologists." In the last edition of "Biological Stains" (Conn, et al, 1940) one finds only a few references to the use of acid dyes for staining bacteria. Those mentioned are mainly fluorescein dyes, but include also brilliant yellow as counterstain for acid fast bacteria, fast acid blue R for bacteria in soil, light green and eosin in Wright's stain for capsules and spores. During the past three years the writer has used several acid dyes for routine staining of bacteria, and to some extent in classes in general bacteriology. The results have been so satisfactory that it seems desirable to publish an account of the uses, formulas of solutions, staining procedures and advantages of these dyes.

Conn (1918) reported using a 1% solution of rose bengal in 5% aqueous phenol for staining bacteria in soil. Three years later (1921) he emphasized the fact that rose bengal has great affinity for bacterial protoplasm, but not for slime or debris with which the cells may be surrounded; and also that it is unusually well adapted for revealing structure in bacterial cells. He proposed that it be employed as a general bacterial stain. Winogradsky (1924, p. 140)

used erythrosin for soil bacteria. Conn and Holmes (1926) and Conn (1926) investigated erythrosin, rose bengal B and eleven other dyes in the fluorescein group. They listed phloxines, erythrosin Y, and rose bengal as good for staining bacteria. Some improvement in technic was also suggested. A little later Conn and Holmes (1928) recommended the addition of a trace of a salt of Ca, Al, Mg or Pb, especially CaCl_2 , to staining solutions of eosin, erythrosin, phloxine and rose bengal. Romell (1934) published a procedure for staining soil microorganisms with a phenolic solution of fast acid blue R.

It seems, therefore, that of the acid dyes only certain of the fluoresceins have been used to any extent for staining bacteria, and mainly soil organisms. On the basis of my own experience several other acid dyes can be recommended for general staining of bacteria, especially acid fuchsin, fast green FCF, acid fuchsin and fast green mixed, anilin blue w. s., and orseiline BB.

Some advantages of using acid dyes for staining bacteria may be mentioned. The staining solutions listed below are easy to prepare and apparently will keep several months without deterioration. No fixing with heat is needed. Staining is very rapid, particularly with acid fuchsin, fast green and anilin blue w. s. As a general rule slime and debris stain lightly. Slides stained with acid dyes are never messy and require less washing than is usual with basic dyes. It is possible to secure marked differentiation within the vegetative cell or between spore and parent cell, by using one dye as a primary stain and a second one to destain the first and to act as a counter-stain at the same time. It seems also that not only rose bengal, as stated by Conn (1921), but most of the acid dyes used here are well adapted for revealing bacterial cell structure. Apparently the staining is quite permanent with anilin blue w. s., fast green or acid fuchsin used singly or mixed, fast acid blue R and orseiline BB. Slides of large spirals in a mixed culture stained with fast green in 1937 show no evidence of fading; and others of about the same age stained with other dyes are still deeply colored. The writer has not tested the fluorescein dyes for permanency.

The following dyes were used in this work: acid fuchsin, Coleman and Bell, uncertified; anilin blue w. s., Cert. No. NK3; erythrosin, Cert. No. CEr-3; fast acid blue R, National Aniline and Chemical Company; fast green FCF, Coleman and Bell, uncertified; light green SF yellowish, Cert. No. CL-8; orseiline BB, Grüber; phloxine, Cert. No. NPh6 and NPh7; rose bengal, Grüber.

The organisms stained included mainly *Alcaligenes viscosus*, *Bacillus cereus*, *Bacillus megatherium*, *Bacillus mycoides*, *Bacillus subtilis*,

Bacterium coli, clostridia in mixed cultures, *Pseudomonas pyocyanus*, *Rhizobium meliloti*, *Rhizobium trifolii*, *Rhodococcus roseus*, *Saccharomyces ellipsoideus*, *Sarcina lutea*, *Serratia marcescens*, *Spirillum rubrum*, spirochaetes, etc. from the mouth, *Staphylococcus citreus*, and *Streptococcus lactis acidi*.

The pure cultures of bacteria listed have been used in class work for a number of years at the University of Missouri. Staining and physiological reactions indicate that they are correctly identified. So far as is known they were received from the following sources: *Alcaligenes viscosus*, *Bacillus cereus* and *Spirillum rubrum*, American Type Culture Collection; *Bacillus megatherium*, *Bacterium coli*, American Museum of Natural History; *Rhodococcus roseus*, Army Medical Museum; *Streptococcus lactis*, isolated at the University of Missouri. *Rhizobium meliloti* and *Rhizobium trifolii* were obtained directly from fresh nodules on living hosts. The exact source of the other six species of bacteria is unknown. *Saccharomyces ellipsoideus* was received in 1925 and seems to be correctly identified.

FORMULAE

| | | |
|--------------------|-------------------------------------------|----------|
| <i>Solution A.</i> | Phenol, 5% aqueous. | 30 cc. |
| | Glacial acetic acid, 20% aqueous. | 8-10 cc. |
| | Ferric chloride, 30% aqueous. | 4 cc. |

Add a 1% aqueous solution of any one of the following dyes in the quantities indicated: acid fuchsin (1-2 cc.); anilin blue w. s. (2-8 cc.); fast green FCF (2-8 cc.); light green (2-8 cc.); fast acid blue R (15 cc.) omitting ferric chloride; orselline BB (8 cc.) omitting ferric chloride.

| | | |
|--------------------|-----------------------------|---------|
| <i>Solution B.</i> | Phenol, 5% aqueous. | 100 cc. |
|--------------------|-----------------------------|---------|

To solution B add 1 gram of rose bengal, erythrosin or phloxine, previously dissolved in about 5 cc. of distilled water. Then add drop by drop enough (6-8 drops) 20% aqueous acetic acid to cause a trace of cloudiness. Finally add 20 to 50 cc. of 95% alcohol. This seems to improve the solution and if a slight cloudiness has developed it should disappear; if not add a trace of ammonium hydroxide in 95% alcohol.

Mixed solutions of dyes. Acid fuchsin (solution A plus dye) may be added to either fast green (A plus dye) or light green (A plus dye) in the proportion of 1 part acid fuchsin to 4 parts of the other; or enough acid fuchsin may be added to the green dye to produce a deep reddish purple color. This mixture stains a greenish blue to nearly purple. It has considerable selective action too, especially if applied for only a few seconds. Spores, for example, may stain light pink and the mother cells green to purple.

In general aqueous or phenolic solutions of acid fuchsin, anilin blue, fast green, or light green stain bacteria very lightly or scarcely at all. The use of the ferric chloride is therefore very important but the part it plays is unknown.

STAINING PROCEDURES

In the staining procedures described here it will be understood that whenever a particular dye is mentioned it has been made up according to one of the formulae given above.

Vegetative cells. In general the procedure for staining vegetative cells of bacteria with acid dyes is to prepare a smear in a drop of water, dry without heating, stain 10 seconds to 1 minute, wash with water and dry. A longer time (2-3 minutes) is better with the fluorescein dyes and with fast acid blue R. Staining is very good and clear-cut with fast green, acid fuchsin, anilin blue w. s., fast green and acid fuchsin mixed, or with acid fuchsin followed by fast green (about 5 seconds). The last procedure gives results similar to those with acid fuchsin and fast green mixed, but by varying the time properly one may obtain more differentiation. This differentiation may frequently be improved by treating 1 minute with Gram's iodine and washing.

If fast acid blue R, orseiline BB, rose bengal, phloxine or erythrosin is used, staining is lighter than with the dyes listed above, but still very satisfactory for most purposes. Staining with these dyes is usually decidedly improved if the bacterial films are first treated 2-3 minutes with solution A, washed with water and then stained. Orseiline BB or rose bengal is to be preferred to phloxine or erythrosin. A *very rapid* (1-2 seconds) counterstain with fast green following the red dyes sometimes brings about considerable differentiation.

One of these dyes may be used to destain another and counterstain at the same time. Fast green, for example, will destain the fluorescein dyes and orseiline very rapidly, and acid fuchsin more slowly. Rose bengal or orseiline will destain fast green, anilin blue w. s., or fast acid blue R in 15 seconds to 1 minute.

The bacteria in nodules of legumes are surrounded by considerable slime. Stained preparations much superior to those made with basic dyes may be obtained by using acid dyes as follows: Make a smear directly from a crushed nodule with or without water. After drying, stain 10-30 seconds with acid fuchsin, wash, stain with fast green 3 seconds and wash. Another satisfactory procedure is to treat 2-3 minutes with solution A, wash, stain 1-2 minutes with orseiline, wash, counterstain with fast green 2 seconds and wash. Using either of these procedures the bacteria will be stained red and the background (slime) green with a clear zone around each cell. For class work material from nodules of either red clover or sweet clover proves to be excellent.

In acid-fast staining, methylene blue is commonly used as a counter-

stain. In class work acid fuchsin and fast green mixed, or else fast green alone has been used for counterstaining. This gives a very sharp contrast since the acid fast organisms appear red and others green.

If carbol fuchsin is used as a counterstain when one uses Hucker's or other procedures for Gram staining, it may withdraw considerable of the gentian violet or crystal violet from Gram positive bacteria, even in 3-5 seconds. This destaining action may be avoided by counterstaining with rose bengal, phloxine or erythrosin, even when staining is prolonged as much as a minute.

Spores. Spores of bacteria may be demonstrated very well with acid dyes. Young spores will stain deeply but mature or old ones only lightly. The cells containing spores, however, can always be stained by the following technic to give a sharp contrast: Treat smears 3 minutes with solution A; wash with water, stain 3-5 minutes with acid fuchsin or fast green, singly or mixed, orseiline or rose bengal; wash with water and counterstain with fast green 2-4 seconds after orseiline, 1-2 seconds after rose bengal, and 5-8 seconds after acid fuchsin; or after fast green counterstain 10-30 seconds with rose bengal (50% alcoholic) or orseiline.

Spores of *Bacillus cereus*, *B. megatherium*, *B. mycoides* or *B. subtilis*, when stained with acid dyes show well various stages in germination. Transfers are made from old spore-bearing cultures to agar slopes and incubated at room temperature for 3-6 hours. Then smears are made and stained as described for vegetative cells, with acid fuchsin 1-2 minutes followed by fast green 5-10 seconds. When stained in this way cells from spores that have just germinated are red while older cells are stained greenish purple.

Instead of growing the bacteria on agar slopes, drops of melted agar may be placed on clean slides and allowed to harden. Spot inoculations from a spore suspension in water may be made with a loop and the slides incubated in a moist chamber. The agar films are then dried and stained as above, or with fast green 10-30 seconds, or with orseiline or rose bengal 1-2 minutes, washed and dried. It is possibly still better to stain with fast green, wash and counterstain with rose bengal for 30 seconds. This results in young germinating spores staining green and other cells pink to purplish red.

Capsules and Sheaths. Another use of acid dyes is for demonstrating capsules or sheaths of bacteria. Films may be made in the usual way; or if there is little slime present it is best first to spread a *very thin* layer of albumen fixative (Mayer's) on the slide, then to add a drop of water and to make a smear. If albumen fixative is

used, one should treat the preparation after drying with absolute alcohol and dry to coagulate the albumen. In either case staining should be with acid fuchsin, or with acid fuchsin and fast green mixed; or the film may be treated with solution A, washed, and stained with orseiline or rose bengal.

Background. A stained background is often desirable in studying bacteria. One may use negative preparations made with aqueous solutions of nigrosin, nigrosin mixed with acid dyes, or Congo red, etc. In such preparations the bacteria are either not stained or only slightly stained. If a smear is made in a *thin* layer of albumen fixative, dried, treated with absolute alcohol, stained with acid fuchsin for 10–30 seconds, washed, and then counterstained 2–4 seconds with fast green, or with acid fuchsin and fast green mixed, and washed, most of the bacteria are stained red and the background is green. Smears made in a *thin* layer of Congo red may be dried and stained with acid fuchsin in solution A. This results in red cells in a blue background.

Glycogen. If 3 or 4 beans are covered with about 2 inches of water in a preparation dish and allowed to incubate for 2–6 days, many clostridia will be found inside and around the decaying seeds. This is very good material for demonstrating the occurrence of glycogen, as well as stages in spore formation, in bacteria. A smear may be made on a slide, dried, stained very rapidly with orseiline, rose bengal, or fast green, washed, treated with Gram's iodine for 1 minute, washed and dried. The dye stains the part of the cell that is free from glycogen, while the glycogen appears a reddish brown. Depending on age the whole cell, or all but a small part of it at one end, may give the reaction for glycogen. If a young spore is present in the cell only that part between the spore and one end of the cell will give the glycogen test.

Yeasts. The mature spores of yeasts are easier to stain than those of bacteria, and stain readily with acid dyes. Spores of *Saccharomyces ellipoideus* develop rather abundantly in cultures grown for a week or more at room temperature and then kept in a refrigerator for several months. Or they will develop in two weeks or less on agar prepared according to the following formula (Maneval, 1924): water, 1000 cc.; Liebig's beef extract, 3 g.; sodium chloride, 5 g.; dextrose, 2.5 g.; agar, 15–20 g. The spores may be stained with acid fuchsin or fast green; or with orseiline or rose bengal after treatment for 3 minutes in solution A. Then they may be washed with water, dried, and a *very thin* layer of 1% aqueous nigrosin applied. Such preparations show the vegetative cells and spores

stained and the asci colorless. If, however, a counterstain is used before applying nigrosin, fast green (2-8 seconds) for the red dyes or rose bengal (20-30 seconds) for the green, the vegetative cells will be stained with the counterstain.

Saccharomyces ellipoideus is a favorable organism for demonstrating germination and conjugation of spores. If transfers are made from old cultures containing an abundance of spores to potato dextrose agar slopes, and incubated at room temperature for 5-7 hours, many spores will germinate. Or spot inoculations on hardened drops of agar on slides as described above for bacteria, may be made and, after incubation in a moist chamber, treated as follows: stain with acid fuchsin and fast green mixed, or with acid fuchsin followed by fast green, wash, dry, apply a *thin* layer of aqueous nigrosin and dry. The ascospores, as described by Marchand (1913), may swell and develop vegetative cells directly; or they may enlarge and develop beaks which unite forming conjugation tubes, so that when two ascospores have conjugated they appear somewhat like a capital U or heart-shaped. When these resulting zygosporae germinate, buds may form anywhere on them but most frequently on the conjugation tube.

Acid dyes have been used for some time in lacto-phenol mounts of various kinds of organisms, especially fungi. It is evident from the staining procedures and results described here that a number of acid dyes may be used also as general bacteriological stains, and that for certain purposes they are to be preferred to basic dyes. Actually it is possible to perform quite satisfactorily most or all of the staining described here with only two acid dyes, acid fuchsin and fast green.

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AMYL ACETATE AS A CLEARING AGENT FOR EMBRYONIC MATERIAL

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ABSTRACT.—Amyl acetate is soluble in 95% alcohol and hot paraffin and produces no hardening in objects exposed to its action for prolonged periods. It may be advantageously employed as a general clearing agent and is especially recommended for refractory material. The following schedule has proven satisfactory for whole frog embryos and young tadpoles: 45 minutes to 1 hour in 95% alcohol, 24 hours in amyl acetate, rinse in toluene, 15 minutes each in three changes of paraffin, imbed. Material so treated may be sectioned at 5 μ with comparative ease.

All of the more commonly used clearing agents produce perceptible hardening, even in tissues exposed to their action for the minimum time. This hardening effect is especially objectionable in the case of highly refractory objects such as amphibian embryos which, in the early stages, contain large amounts of yolk. Yolk granules may be rendered hard enough to injure the knife. Furthermore, some clearing fluids (e.g. dioxan) are chemically active and cannot be used after certain fixatives or interfere with subsequent staining reactions.

The use of amyl acetate, suggested by Barron¹ seems to be free from these objections. This fluid is sufficiently soluble in hot paraffin to permit rapid infiltration and is miscible with 95% alcohol. The use of absolute alcohol with attendant shrinkage and hardening is thus avoided. In addition, no hardening whatever is produced by the most prolonged treatment with amyl acetate. Amphibian eggs have been stored in this fluid for over a year without undergoing any changes in sectioning properties. In fact, in desirability as a storage medium, amyl acetate far surpasses the commonly employed 70% alcohol which may produce noticeable hardening in a week's time.

The writer has found the following procedure to be entirely satisfactory for the treatment of whole frog embryos from the earliest stages thru the late tailbud stage: Following fixation, the embryos are rinsed in water and then transferred directly to 70% alcohol. After about an hour in this solution, the vitelline membranes, if still present, are removed and the embryos transferred directly to 95% alcohol and allowed to remain in this fluid for from 45 minutes to 1 hour—no longer. They are then transferred to pure amyl acetate where they may remain indefinitely, altho a 24-hour treatment (perhaps less) is ample. Each of the alcohols and the amyl acetate should be changed at least once. The amyl acetate is then

¹Barron, D. H. 1934. Amyl acetate: A useful solvent for embedding masses. *Anat. Rec.*, 59, 1-8.

decanted, the vial containing the embryos is filled with toluene, and the embryos are *immediately* transferred to pure melted paraffin by means of a wide-mouthed pipette. The purpose of the toluene is merely to rinse off the excess amyl acetate which, being relatively involatile, would otherwise accumulate in the paraffin bath. Any other volatile liquid would serve just as well. The paraffin should have a melting point of about 58° C. and the temperature of the oven should be such that a part of the paraffin remains unmelted. The tissue should be allowed to remain for 15 minutes in each of 3 changes of paraffin before imbedding. In no case should the embryos be exposed to this temperature for more than 1 hour. No advantage is gained by inserting in this series a bath consisting of a mixture of paraffin and the clearing agent.

When subjected to this treatment, the embryos remain so soft at the time of imbedding that extreme care must be taken not to injure them during the process of orientation. The instrument used in orienting them should be well heated in a flame before immersion in the paraffin. It is probable that the difficulties usually ascribed to "crystallization" of the paraffin are frequently due to the introduction of minute air bubbles into the paraffin by the use of an insufficiently warmed orienting needle. At any rate, if the needle has been heated until it smokes, the block may be allowed to solidify at room temperature without adversely affecting its sectioning properties. The cutting properties of the paraffin may be improved by the addition of a little gum rubber or one of the prepared compounds containing this substance.

During the past year, a number of other workers in this laboratory have used amyl acetate for clearing a variety of tissues following many special as well as general fixatives and preceding a large number of staining technics. In all cases, the results have been highly satisfactory, the form and staining properties of zymogen granules, mitochondria, etc. being unaffected. It seems to be particularly suitable for clearing large objects which would be excessively hardened in the time required for the penetration of absolute alcohol and the usual clearing agents. Since amyl acetate does not produce optical clearing, the time required must be determined empirically as for the alcohols and the paraffin. Since there is no maximum period, however, this is not a difficult procedure.

The odor of amyl acetate may become quite disagreeable after prolonged inhalation of the vapor. Possible adverse physiological effects have not been noticed but it would probably be wise to observe the same precautions in the handling of this reagent as in the handling of chloroform, dioxan, etc. Waste amyl acetate should be kept in a stoppered bottle, as it is rather difficult to flush down the drain.

ALIZARIN RED S AND TOLUIDINE BLUE FOR DIFFERENTIATING ADULT OR EMBRYONIC BONE AND CARTILAGE

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ABSTRACT.—This technic has been successfully employed by the author for staining, *in toto*, the bones and cartilage of mature specimens of *Urodela* and the developing bone and cartilage of the embryonic human, cat, pig and rat. The differential staining is accomplished by using a modification of Dawson's method of staining bone with alizarin red S following a toluidine blue solution specific for cartilage. Specimens are fixed in 10% formalin, stained one week in a solution of .25 g. of toluidine blue in 100 cc. of 70% alcohol, macerated 5 to 7 days in a 2% KOH solution, counterstained for 24 hours in a 0.001% solution of alizarin red S in 2% aqueous KOH, dehydrated in cellosolve and cleared in methyl salicylate. In the adult and embryonic forms thus treated the soft tissues are cleared while the osseous tissue is stained red, the cartilage blue.

INTRODUCTION

A number of authors have described procedures for staining the bones and clearing the soft tissues of embryos and small animals *in toto*. Among these workers there exists a difference of opinion with regard to the fixative to be employed. Dawson's (1926) original technic indicated the use of 95% alcohol and with this Lipman (1935) agrees. Gray (1929) employs iodine-alcohol followed by absolute alcohol. The present author, however, has found that tissue fixed in 10% formalin for at least one week can be better controlled during subsequent maceration in KOH. The macerative effect of KOH on tissue which has been fixed in alcohol is comparatively rapid, but there is constant danger that such tissue will too quickly reach the point of total maceration. Such destruction rarely occurs in formalin-fixed specimens, nor is a constant check required to prevent it.

The use of methyl salicylate for clearing tissue (first suggested by Spalteholz, 1914) has two slight disadvantages. Tissue so cleared has a tendency to turn brown over a period of years. This may be remedied by transferring the specimen to a fresh lot of the oil and reclearing. Secondly, the tissue will undergo a slight amount of shrinkage; not enough, however, to affect the relations of the skeletal

parts in any appreciable manner. Cumley, Crow and Griffin (1939) have cleared embryos, previously macerated in KOH, in toluol followed by storage in anise oil saturated with naphthalene. Schultze (1897) macerated his specimens in KOH and cleared them in glycerin. Specimens thus cleared are easily dissected, for the tissues remain soft and pliable and undergo no shrinkage. However, glycerin will render the soft tissues only semitransparent.

PROCEDURE

1. *Preparation and fixation.* If the bone and cartilage are to be submitted to detailed microscopic observations the specimen should be completely eviscerated, the contents of the thorax and abdomen being removed thru a small incision in the abdominal wall. This procedure is not necessary for specimens which are to be used as museum preparations. The specimen is washed in plain tap water and immediately fixed in a 10% formalin solution for at least one week.

2. *Cartilage staining.* The specimen is washed for 24 hours in 250 cc. of 70% alcohol to which 10 drops of NH₄OH have been added. It is then stained one week in a toluidine blue solution prepared as follows: to 100 cc. of 70% alcohol add 2 cc. of 0.5% HCl and 0.25 g. of toluidine blue.¹ The mixture is allowed to stand for 24 hours, at the end of which time it is filtered and stored in a tightly corked container. The specimen is next hardened and destained for 72 hours in four changes of 95% alcohol.

3. *Maceration.* The specimen is macerated in several changes of a 2% aqueous solution of KOH, the length of time depending upon the size of the animal. The average time for early mammalian embryos is from 5 to 7 days, or until the bones of the limbs become visible thru the macerated integument. This process is greatly facilitated by exposure to sunlight or ultra-violet rays.

4. *Bone staining.* The specimen is transferred to a fresh solution of 2% KOH to which is added, by drops, a saturated alcoholic solution of alizarin red S,¹ enough to turn the hydroxide a deep wine red. At the end of 24 hours the bones should appear well stained. If the specimen has been insufficiently macerated the soft tissues will appear slightly stained. In this event the specimen may be quickly destained in acid alcohol (1% H₂SO₄ in 95% alcohol).

5. *Dehydration, clearing and storage.* Dehydration is accomplished by running the specimen thru 3 changes of cellosolve² of 6 hours each. For small embryos this time may be considerably

¹Coleman and Bell dyes (not certified) were used in this technic.

²Manufactured by the Carbide and Chemicals Corp., New York City, N. Y.

reduced. (The alcohol series may be substituted for this step, 50%, 80% and 90%, followed by 3 changes of benzene.) The best medium for clearing the specimen after dehydration is methyl salicylate (synthetic oil of wintergreen) which possesses essentially the same index of refraction as the tissues to be cleared. From the cellosolve the specimen is transferred to solutions of 25%, 50% and 75% of methyl salicylate in cellosolve, for 24 hours each. This step must be carefully observed in order to prevent undue shrinkage of the tissues. It is then transferred to pure methyl salicylate for permanent storage.

In animals thus treated the soft tissues are rendered transparent while the osseous tissue is stained a deep red, the cartilage a dark blue. In addition, the intensity of the stains serve to indicate the relative amounts of ossification and chondrogenesis which have taken place. The bone or cartilage may be stained separately by omitting step 2 for bone or step 4 for cartilage. Excellent permanent preparations may be made of parts such as limbs, hands or heads by mounting them in Canada balsam on a deep depression slide. The tissues of such preparation are cleared well enough to permit detailed microscopic observations of the gross osseous and ~~cartilaginous~~ structures.

For those who might prefer clearing in glycerin the author suggests the following modification of the technic presented in this paper: Omitting step 5, transfer the specimen directly from the KOH-alizarin solution into a series of 50%, 70% and 80% glycerin solutions for 24 hours each. It may then be stored in pure glycerin where the soft tissues will continue to clear for several days. Should further clearing be desired the specimen is transferred from the glycerin into cellosolve and treated as per step 5.

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THE REACTION OF CERTAIN STAINS WITH BACTERIA¹

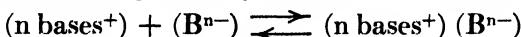
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ABSTRACT.—In view of the controversy over the contention that staining is the result of physical or chemical affinity between a stain and a bacterial cell, this paper is presented to show some recent data which support the idea that staining is an adsorption exchange process chemical in nature.

It is a well established fact that when a stain is added to a suspension of bacteria, a certain amount of the stain is removed from solution and concentrated in and about the cell. The forces that attract the stain to the cell and bind it there have been described as both chemical (Stearn and Stearn, 1929, 1930) and physical (Holmes, 1929) in nature.

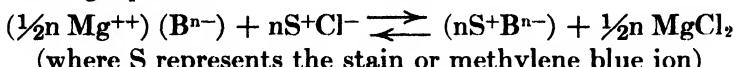
The present work is an attempt to show that the staining of a bacterial cell occurs as a result of an adsorption exchange process which approaches the magnitude of stoichiometrical proportions. The data accumulated seem to indicate that the reaction is of a chemical nature.

The mechanism of exchange adsorption may be visualized by the use of equations; for example, a suspension of negatively charged bacteria should attract positively charged ions (McCalla, 1940, a, b).



(B represents the bacterial cell with an unknown number of negative ionic valences, n.).

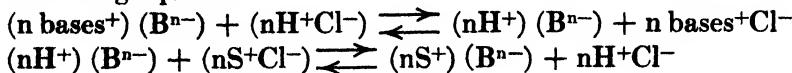
As represented by this equation the attraction of the negatively charged bacterial cell for the positively charged bases should produce a neutral system; that is, the bases should be adsorbed by the cell. If a stain is adsorbed at the same positions as the bases, it should be possible to replace an adsorbed base when the stain is added. For example, if methylene blue chloride were added to a suspension of bacteria saturated with adsorbed Mg, at any given concentration, it should result in a displacement of the Mg by the stain, according to the following equation:



It should be possible to measure the displaced Mg chemically and show a stoichiometrical relationship between the methylene blue

¹Contribution No. 197, Department of Bacteriology.

adsorbed and the Mg displaced. This adsorption exchange view may be tested by an additional method. If normal bacteria with different bases in the adsorption sphere are transformed into a system with predominantly adsorbed hydrogen, it should be possible to add methylene blue chloride and replace the adsorbed H⁺, according to the following equation:



The replacement of the adsorbed H⁺ should result in an increase in H⁺ concentration which may be measured.

EXPERIMENTAL

The following stains—methylene blue,² medicinal, 88% purity; crystal violet,³ 88%; basic fuchsin,³ 91%; safranin 0,³ 94%; phloxine—were prepared as 0.01 M aqueous solutions, except basic fuchsin which was used in an 0.008 M concentration. All stock solutions were kept in paraffined flasks. The adsorption displacement of cations by the use of basic stains was determined with five different organisms, *Staphylococcus aureus*, a spore forming bacillus, and three strains of *Escherichia coli*. The cultures were grown on salts agar⁴ in large bottles for one to two days at 37°C. At the end of this period the cells were suspended in distilled water and washed free of dissolved materials by repeated use of distilled water and a Sorvall centrifuge. Since in general staining the organisms are usually dead, the cells were killed by heating at a temperature of 100°C. for three to five minutes. Such cells when stained appeared normal.

MAGNESIUM DISPLACEMENT WITH METHYLENE BLUE

It was assumed in the preceding discussion that if Mg were held by bacteria in the adsorption sphere, it should be replaced in staining. To insure the presence of adsorbed Mg, *Escherichia coli* was grown on salts agar containing five grams of MgSO₄ per liter. *Staphylococcus aureus* cells were prepared by growing in salts agar and then adding a dilute solution of MgSO₄ to the washed cells. These cells were allowed to stand for about thirty minutes before washing free of dissolved Mg. To washed aliquots of the suspension, known quantities of 0.01 M methylene blue chloride were added and carefully mixed. The cells were then thrown out of suspension by use of the centrifuge. The supernatant liquid from these cells presumably containing the

²Certified by the Commission on the Standardization of Biological Stains.

³Found satisfactory by the Commission on Standardization of Biological Stains.

⁴The medium contains the following ingredients: potassium citrate, 1.0 g.; diammonium phosphate, 0.5 g.; potassium bicarbonate, 0.5 g.; dipotassium phosphate, 1.5 g.; peptone, 20.0 g.; dextrose, 0.5 g.; agar agar, 25.0 g.; distilled water to make 1000 ml. This medium was adjusted to neutral reaction with NaOH.

displaced Mg and some unadsorbed methylene blue was evaporated and ignited. The Mg was determined by the method of Baver and Bruner (1939). Controls, one with bacterial cells to which no methylene blue was added and another with only methylene blue, were carried along with each determination. The data given in Tables 1 and 2 are representative and show clearly that when methylene blue was adsorbed by *Escherichia coli* and *Staphylococcus aureus* it replaced Mg with which the cells had previously been saturated. Only a trace of Mg was present in the supernatant liquid from the magnesium-saturated bacteria to which no methylene blue was added, and none was present in the methylene blue.

TABLE 1. THE DISPLACEMENT OF MAGNESIUM FROM *Escherichia coli* WITH METHYLENE BLUE

| Trials | Amount of Mg in Methylene Blue | m.e.* Mg replaced/100 g. Bacteria† | | m.e. of Methylene Blue adsorbed per 100 g. of Bacteria |
|--------|--------------------------------|------------------------------------|---------------------------|--------------------------------------------------------|
| | | Water+Bacteria | Methylene Blue + Bacteria | |
| 1 | 0.0 | Trace | 21.4 | 22.8 |
| 2 | 0.0 | " | 38.5 | 60.8 |
| 3 | 0.0 | " | 39.3 | 52.2 |
| 4 | 0.0 | " | 60.0 | 64.0 |
| 5 | 0.0 | " | 30.6 | 48.9 |

TABLE 2.—THE DISPLACEMENT OF MAGNESIUM FROM *Staphylococcus aureus* WITH METHYLENE BLUE

| Trials | Amount of Mg in Methylene Blue | m.e.* Mg replaced/100 g. Bacteria† | | m.e. of Methylene Blue adsorbed per 100 g. of Bacteria |
|--------|--------------------------------|------------------------------------|---------------------------|--------------------------------------------------------|
| | | Water+Bacteria | Methylene Blue + Bacteria | |
| 1 | 0 | Trace | 17.3 | 30.5 |
| 2 | 0 | Trace | 10.9 | 24.1 |

*m.e. refers to milligram equivalents.

†Expressed as weight of cells dried at 105°C.

HYDROGEN REPLACEMENT WITH BASIC STAINS

Stearn and Stearn (1929) definitely showed that when basic stains, adjusted to a certain pH-value, were added to protein solutions of peptone, gelatin and nucleic acids, also adjusted to the same pH-value, they became more acid. Joseph (1940) from his observations on previous studies of pectin concluded that H⁺ was adsorbed by this system. The adsorption was determined by the change in pH when sulphuric acid was added to the pectin system.

To determine whether basic stains would replace H⁺ adsorbed by bacteria, a sufficiently dilute solution of HCl was added to washed

cells of bacteria to give a pH-value of approximately five and the unadsorbed H-ions were removed by washing. The pH value of the stain was adjusted above that of the bacterial suspension. Increasing amounts of the basic stains were then added to 10 ml. aliquots of bacterial suspension treated with H⁺ (H-bacteria) along with sufficient distilled water to make the volume the same in all instances. The increase in H⁺ was measured with both glass and quinhydrone electrodes. All data reported were obtained with the glass electrode.

Some of the typical data are reported in Tables 3, 4, and 5. An

TABLE 3. THE DISPLACEMENT OF ADSORBED HYDROGEN WITH CRYSTAL VIOLET AND SAFRANIN O FROM BACILLI

| ml. of H-bacteria | ml. of Stain | ml. of Water | pH of H-bacterial Suspension | | | |
|----------------------|-----------------|-----------------|--------------------------------|-------------------|----------------------------|-------------------|
| | | | Treated with Crystal Violet | | Treated with Safranin O | |
| | | | Actual pH | Decrease in pH | Actual pH | Decrease in pH |
| 10.0 | 0.0 | 5.00 | 6.00 | 0.0 | 6.00 | 0.0 |
| 0.0 | 5.0 | 10.00 | 6.55 | 0.0 | 7.00 | 0.0 |
| 10.0 | 0.1 | 4.90 | 5.68 | 0.92 | 5.60 | 0.40 |
| 10.0 | 0.25 | 4.75 | 5.60 | 0.40 | 5.55 | 0.45 |
| 10.0 | 0.50 | 4.50 | 5.45 | 0.55 | 5.45 | 0.55 |
| 10.0 | 1.00 | 4.00 | 5.35 | 0.65 | 5.28 | 0.72 |
| 10.0 | 2.00 | 3.00 | 4.92 | 1.08 | 5.00 | 1.00 |
| 10.0 | 3.00 | 2.00 | 4.58 | 1.42 | 4.82 | 1.18 |
| 10.0 | 4.00 | 1.00 | 4.38 | 1.62 | 4.82 | 1.18 |
| 10.0 | 5.00 | 0.00 | 4.35 | 1.65 | 4.82 | 1.18 |

TABLE 4. THE DISPLACEMENT OF HYDROGEN FROM *Escherichia coli* WITH METHYLENE BLUE AND CRYSTAL VIOLET

| ml. of H-bacteria | ml. of Stain | ml. of Water | pH of H-bacterial Suspension | | | |
|----------------------|-----------------|-----------------|--------------------------------|-------------------|--------------------------------|-------------------|
| | | | Treated with Methylene Blue | | Treated with Crystal Violet | |
| | | | Actual pH | Decrease in pH | Actual pH | Decrease in pH |
| 10.0 | 0.0 | 5.00 | 4.90 | 0.00 | 5.60 | 0.00 |
| 0.0 | 5.00 | 10.00 | 5.90 | 0.00 | 6.20 | 0.00 |
| 10.0 | 0.10 | 4.90 | 4.80 | 0.10 | 5.45 | 0.15 |
| 10.0 | 0.25 | 4.75 | 4.55 | 0.35 | 5.32 | 0.28 |
| 10.0 | 0.50 | 4.50 | 4.40 | 0.50 | 5.13 | 0.47 |
| 10.0 | 1.00 | 4.00 | 4.18 | 0.72 | 4.90 | 0.70 |
| 10.0 | 2.00 | 3.00 | 4.10 | 0.80 | 4.50 | 1.10 |
| 10.0 | 3.00 | 2.00 | 4.10 | 0.80 | 4.55 | 1.05 |
| 10.0 | 4.00 | 1.00 | 4.10 | 0.80 | 4.50 | 1.10 |
| 10.0 | 5.00 | 0.00 | 4.10 | 0.80 | 4.50 | 1.10 |

examination of the data reveals that in every case there was an increase in H^+ when a basic dye combined with a bacterial system. It is also evident that there was a maximum acidity, and that any addition of dye beyond this range did not result in any further increase in acidity.

TABLE 5. THE DISPLACEMENT OF HYDROGEN WITH BASIC FUCHSIN FROM *Staphylococcus aureus* AND *Escherichia coli*

| ml. of H-bacteria | ml. of Stain | ml. of Water | pH of H-bacterial Suspension | | | |
|----------------------|-----------------|-----------------|------------------------------|-------------------|-------------------|-------------------|
| | | | <i>Staph. aureus</i> | | <i>Esch. coli</i> | |
| | | | Actual pH | Decrease in pH | Actual pH | Decrease in pH |
| 10.0 | 0.00 | 5.00 | 6.00 | 0.00 | 4.50 | 0.00 |
| 0.0 | 5.00 | 10.00 | 6.10 | 0.00 | 6.20 | 0.00 |
| 10.0 | 0.10 | 4.90 | 5.80 | 0.20 | 4.55 | +0.05 |
| 10.0 | 0.25 | 4.75 | 5.70 | 0.30 | 4.38 | 0.12 |
| 10.0 | 0.50 | 4.50 | 5.50 | 0.50 | 4.25 | 0.25 |
| 10.0 | 1.00 | 4.00 | 5.25 | 0.75 | 4.00 | 0.50 |
| 10.0 | 2.00 | 3.00 | 5.00 | 1.00 | 3.80 | 0.70 |
| 10.0 | 3.00 | 2.00 | 4.65 | 1.35 | 3.75 | 0.75 |
| 10.0 | 4.00 | 1.00 | 4.25 | 1.75 | 3.72 | 0.78 |
| 10.0 | 5.00 | 0.00 | 4.15 | 1.85 | 3.72 | 0.78 |

TABLE 6. THE INFLUENCE OF AN ACID DYE UPON THE pH OF *Escherichia coli*

| ml. of H-bacteria | ml. of Stain | ml. of Water | pH of H-bacterial Suspension treated with Phloxine | |
|----------------------|-----------------|-----------------|-------------------------------------------------------|----------------|
| | | | Actual pH | Increase in pH |
| 5.0 | 0.00 | 2.00 | 4.90 | 0.00 |
| 5.0 | 0.10 | 1.90 | 6.08 | 0.78 |
| 5.0 | 0.25 | 1.75 | 6.20 | 0.90 |
| 5.0 | 0.50 | 1.50 | 6.23 | 0.93 |
| 5.0 | 1.00 | 1.00 | 6.15 | 0.85 |
| 5.0 | 2.00 | 0.00 | 6.00 | 0.70 |
| 0.0 | 5.00 | 2.00 | 5.30 | 0.00 |

THE INFLUENCE OF AN ACID STAIN ON THE pH-VALUE OF *ESCHERICHIA COLI*

Since acid stains act as anions (Conn, 1940) they should have the opposite effect on the pH-value of a suspension of H-bacteria to that of basic stains. The influence of phloxine upon the pH-value of suspended *Escherichia coli* cells is shown in Table 6. It is evident that the pH-value increased as the quantity of phloxine increased up to a certain maximum and that further additions of the stain did not increase the pH-value. When 1 ml. or more of phloxine was added to the bacterial suspension the pH-value decreased. This may be

attributed to having an excess of the stain which has a pH-value below that of the stain-bacteria mixture.

DISCUSSION

The data collected in this investigation agree with the views set forth in the introduction; namely, that when a stain combines with a bacterial cell it replaces some ion already adsorbed by the cell. This was demonstrated by using bacteria saturated with adsorbed Mg^{++} or H^+ at specific concentrations. When a basic stain was added the adsorbed Mg^{++} or H^+ was released and found in the supernatant liquid, while the stain became adsorbed by the cell. This replacement indicates that a stain is adsorbed at the same position as the inorganic cations. Assuming that stains have a similar mechanism of attachment to a bacterial cell as inorganic cations, this should establish a common basis for evaluating the effect of the two types of substances on bacteria.

CONCLUSIONS

The data obtained in this investigation support these conclusions:

1. The reaction of stains with bacteria is an adsorption exchange process, reaching stoichiometrical proportions. Two methods were used to show that staining is an exchange reaction.
2. Basic stains act as cations, replacing similarly charged ions from the bacterial system.
3. Basic stains decrease while acid stains increase the pH value of a suspension of H-bacteria (i.e. bacteria treated with H^+).
4. From the increase in acidity obtained by displacing H^+ with a basic stain there appears to be a maximum exchange value.
5. Stains appear to react with the bacterial cell at the same positions as do inorganic cations.

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P-AMINODIMETHYLANILINE MONOHYDROCHLORIDE AS AN INDICATOR OF MICROBIAL ACTION ON FATS

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ABSTRACT.—Flooding p-aminodimethylaniline monohydrochloride on fat emulsion agar inoculated with certain types of microorganisms frequently results in marked color changes in the fat globules. It is shown in this paper that the colors result from the increased solubility in fat and fatty acids of this dye as it becomes oxidized. Some of the acids oxidize the dye on contact and therefore color very quickly; fats become colored only when some other agent oxidizes the dye. The characteristic color reactions with certain fats and fatty acids are described for various degrees of oxidation of the dye; this suggests the explanation for the colors observed in the inoculated globules flooded with this dye. A table is included showing the colors in globules of oil that were inoculated with 39 pure cultures of bacteria.

For detection of fat splitting bacteria, Jensen and Grettie (1937) have combined the oxidase test described by Gordon and McLeod (1928) with an unstained oil emulsion agar. With this medium they are able to identify lipolytic organisms by the transparent or translucent zone surrounding the colonies. Those organisms which incite or aid in the production of oxidative rancidity are recognized by their colony color reaction when flooded with dyes of the paraphenylenediamine series.

Castell and Bryant (1939) have observed that at least one of these oxidase detecting dyes, *p*-aminodimethylaniline monohydrochloride, also produces a variety of color changes in fat globules undergoing decomposition. These can be observed under the low power lens of the microscope, and they vary from red, yellow, brown or black to colorless. By examining the action of this dye on triglycerides, fatty acids and other organic compounds, it was shown that the reduced dye solution had little effect on the fats but was readily absorbed and oxidized by the fatty acids, producing shades of color of light brown to black. The color darkened with each decrease in the molecular weights of the acids. It was concluded that the presence of free acid in the decomposing fat globules enabled them to absorb the dye from the surrounding agar medium. Further work, including attempts to discover the reason for green, blue and other color

changes in fat globules inoculated with certain types of bacteria and stained with this indicator, is summarized in this article.

Color Reactions of the Dye. When a fresh 0.5% aqueous solution of this dye is prepared it is almost colorless. On standing, it gradually becomes oxidized to shades of pink rose, dark red and reddish black. It becomes a dark purple to bluish black on further exposure. Up to the point where the solution becomes dark red, it can be reduced again. Once the blue black stage has been reached, however, the color change does not appear to be reversible.

Triolein and olive oil will not absorb the dye in its reduced form. However, as it becomes oxidized to shades of dark red it can be readily absorbed by the fat from the aqueous solution, coloring the oil a deep red. Neither triolein nor olive oil is colored by the dye when it has been oxidized to the non-reversible bluish-black stage.

Oleic acid is colored a light brown or amber color when mixed with the dye in a pale pink form. As the dye is further oxidized until the dark red or purple stage is reached, the depth of brown color increases as oleic acid is added. Mixed with the dye in its dark blue stage this acid remains colorless.

The insoluble saturated fatty acids turn to shades of greyish brown and black when mixed with the pale pink dye solution. The fewer the number of carbon atoms in the acids, the more intense the color reaction. When added to dye solutions oxidized thru shades of dark red, purple and blue, the acids also take on purple and blue shades.

Significance of Color Reactions in Inoculated Fat Globules. The application of these results to the color changes in the fat globules of an inoculated oil emulsion agar suggests the following interpretations:

(1) Red globules under, or surrounding a colony, indicate that the bacterial oxidase has oxidized the dye in the immediate vicinity of the colony, to the stage where it has become fat soluble. When the plate is left exposed to the air after the dye has been added, all the globules will turn red in a few hours. There is no reason to believe that this color change results from any change in the fat. However, it is not unlikely that enzymes actively oxidizing the dye may also aid in the oxidation of unsaturated fats.

(2) The presence of amber or buff colored globules suggest fatty acids, more especially oleic or some similar acid.

(3) Dark brown or black globules unaccompanied by an outer ring of red globules would suggest the presence of caproic, caprylic or capric acids and indicate an oxidase negative colony.

(4) Blue and green globules show the presence of these same saturated acids together with a strongly oxidase positive bacterial colony.

(5) When globules around or under the colonies become colored at the same rate as globules in uninoculated portions of the plate, the bacterial colony neither hydrolyzes the fat nor is oxidase positive.

(6) When the globules remain colorless around a colony long after those in other portions of the plate have become red, the bacterial colony is exerting a strong reducing action on the surrounding medium.

Color Reactions With Pure Cultures of Bacteria. Fat emulsion medium was prepared by shaking olive oil in standard nutrient agar. This was poured into sterile petri plates and when cool, streaked with pure cultures of bacteria. After incubation at 25°C. (77°F.) for four days, the plates were flooded with a freshly prepared 0.5% aqueous solution of *p*-aminodimethylaniline monohydrochloride. After standing for two or three minutes the dye was poured off and the plates were examined microscopically after one-half, one, two and six hours.

Table 1 shows the results of these observations compared with the color reactions of Nile blue sulphate in the same medium with the same organisms.

It will be noted that the results of this dye on the various pure cultures used, agree fairly accurately with those obtained by using Nile blue sulphate as the indicator. With some organisms the globules were colored differently in different areas. The globules surrounding the colonies of the *Pseudomonas* group and *Phytomonas campestris*, were colored a dark red; those under the colony were shades of blue and buff.

Similar plates were examined after 24 days' incubation. Several cultures, especially *Escherichia coli*, *Aerobacter aerogenes* and *A. cloaceae*, which had formerly given negative reactions, turned dark brown when flooded with the dye solution, indicating acid production.

For the time being this dye is not recommended as a substitute for Nile blue sulphate or copper sulphate as an indicator for lipolytic bacteria. However, the dye has been shown by Castell and Garrard (1939) to have an increasingly important place in certain phases of dairy bacteriology and has been already used in conjunction with fat emulsion media by Jensen and Grettie (1937). It also appears to have a value in yielding information not given by ordinary dyes used for detecting lipolysis.

TABLE 1. COLOR REACTIONS IN FAT GLOBULES INOCULATED WITH PURE CULTURES OF BACTERIA AND FLOODED WITH *p*-AMINODIMETHYLANILINE MONOHYDROCHLORIDE AND COMPARED WITH THE COLOR REACTIONS OF THE SAME ORGANISMS WITH NILE BLUE SULPHATE AS AN INDICATOR

| Organism | Red | Blue | Amber colored | Blue with Nile blue sulphate |
|---------------------------------------------|-----|------|---------------|------------------------------|
| <i>Bacillus subtilis</i> | - | - | - | - |
| <i>Bacillus mycoides</i> | - | +? | - | +? |
| <i>Bacillus panis</i> | - | - | - | - |
| <i>Bacillus cereus</i> | - | + | - | + |
| <i>Bacillus graveolens</i> | + | - | - | - |
| <i>Bacillus mesentericus</i> | ++ | - | - | - |
| <i>Staphylococcus aureus</i> | - | - | ++ | + |
| <i>Staphylococcus citreus</i> | - | - | + | - |
| <i>Streptococcus lactis</i> | - | - | - | - |
| <i>Micrococcus conglomeratus</i> | + | - | - | - |
| <i>Diplococcus capsulatus</i> | - | - | - | - |
| <i>Sarcina lutea</i> | - | - | +? | - |
| <i>Sarcina aurantiaca</i> | - | - | - | - |
| <i>Erwinia carotovora</i> | ++ | - | - | - |
| <i>Rhizobium radicicola</i> | ++ | - | - | - |
| <i>Phytomonas campestris</i> | + | + | - | + |
| <i>Achromobacter putrefaciens</i> | ++ | - | - | - |
| <i>Achromobacter lipolyticum</i> | + | + | + | + |
| <i>Pseudomonas fluorescens</i> | ++ | + | + | + |
| <i>Pseudomonas aeruginosa</i> | ++ | + | - | + |
| <i>Pseudomonas fragi</i> | ++ | + | - | + |
| <i>Pseudomonas mucidolens</i> | ++ | + | - | + |
| <i>Alcaligenes viscosus</i> | - | - | + | - |
| <i>Alcaligenes lipolyticum</i> | + | + | - | + |
| <i>Alcaligenes faecalis</i> | ++ | - | - | - |
| <i>Brucella abortus</i> | ++ | - | - | - |
| <i>Eberthella typhosa</i> | - | - | - | - |
| <i>Aerobacter aerogenes</i> | - | - | - | - |
| <i>Aerobacter cloaceae</i> | - | - | - | - |
| <i>Salmonella</i> (9 species) | - | - | - | - |
| <i>Proteus vulgaris</i> | - | - | - | - |
| <i>Escherichia coli</i> | - | - | - | - |

++ strong reaction

+ weak reaction

- negative reaction

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NOTES ON TECHNIC

A NOTE ON THE USE OF SYNTHETIC GLYCEROL (SHELL) IN THE CLEARING OF EMBRYOS *in Toto*.—Recently Williams¹ announced the synthesis of glycerol from petroleum gases which has proven of special interest both commercially and pharmacologically. Williams stressed that the synthetic glycerol (Shell), tested pharmacologically proved to produce no ill effects, despite the fact that allyl chloride and alcohol, both highly toxic materials, were intermediate products in the synthesis. Hart² has substantiated these findings in a pharmacological comparison between synthetic glycerol and glycerol produced from natural oils.

Since natural glycerol is used extensively in certain of the well-known clearing processes on embryos *in toto*, it was of interest to the author to compare the synthetic glycerol (Shell) with that of natural glycerol. The procedure was essentially the same as the well-known Schultz modification of Spalteholtz' technic, with the exception of the staining procedure described below.

Initial clearing of the embryos was done with 5% KOH (with smaller specimens only 1-2% KOH was used to prevent disintegration of the tissues) and required 1 to 2 days, depending upon the size of the specimens. Staining of ossification centers was accomplished with a 0.1% alizarin red S solution (National Aniline) in distilled water. The alizarin red was added, drop by drop, with constant stirring, to a 2% Na₂CO₃ solution, until a uniform reddish-purple mixture was obtained. The specimens were transferred directly from the KOH to the staining mixture. Complete staining usually required from 12 to 24 hours in the above staining medium. This method of staining has proven very satisfactory in all specimens stained for bone preparation, with very little or no fading of the dye, even after several months of permanent mounting in glycerin.

After completion of the staining, the specimen is transferred to a 25% solution of synthetic glycerol (Shell) and allowed to remain in the glycerol from 3 to 5 days (again depending upon the size of the specimen). The author has used embryos of rat, pig and calf, and has found the entire skeletal system plainly visible after a few days immersion in the 25% synthetic glycerol. The specimen is then carried over to a 50% synthetic glycerol for 3-7 days. Final clearing

¹Williams, E. C. 1938. Modern petroleum research. Ind. Eng. Chem., 16, 690.

²Hart, E. Ross. 1939. A pharmacological comparison between synthetic glycerol and glycerol prepared from natural oils. Univ. of Calif. Publ. in Pharmacology, 1, 271-4.

is done in pure synthetic glycerol; the specimen mounted for permanent preservation by the method previously described.³ Precaution is taken to add a small amount of crystalline thymol to all glycerol solutions to prevent contamination by fungi.

Comparison of synthetic glycerol (Shell) with that of natural glycerol in the clearing of embryos *in toto* has proved that the synthetic product removes excess dye from the soft tissues, particularly in 25% synthetic glycerol. Repeated observation with natural glycerol fails to reveal any appreciable removal of excess dye in comparison with the synthetic glycerol, unless alcohol is added to the natural glycerol. Synthetic glycerol (Shell) is equally desirable, if not somewhat superior, in the clearing of embryos *in toto*.

The author wishes to express his sincere appreciation to the Shell Development Company of Emeryville, California, for the generous supply of synthetic glycerol used in this investigation.—CLARENCE W. NICHOLS, JR., Santa Cruz, Calif.

A METHOD FOR MAKING PERMANENT MOUNTS OF PORTIONS OF DECOLORIZED WHOLE LEAVES.⁴—A wide-mouthed bottle is a suitable container to carry out the processes of decolorizing, dehydrating and clearing. The leaves are decolorized by a method previously described by the writer.⁵

After decolorizing, the leaves are dehydrated with ethyl alcohol in the usual manner, and the dehydration completed in carbol-xylol which is made by adding one part phenol crystals to three parts xylol. The leaves are then cleared by treating with two changes of pure xylol.

After clearing, the leaves are removed from the bottle one at a time and placed in a shallow dish of xylene. Small portions of the leaf from 5–10 mm. square are cut out with scissors, mounted on a clean slide in a drop of hyrax and covered with a cover glass. When these preparations are examined under the microscope, the different layers of leaf tissue may be observed by simply raising and lowering the objective.—JAMES C. BATES, Kansas State College, Manhattan, Kansas.

⁴Nichols, C. W., Jr. 1940. A simple method for mounting embryological material. *Stain Techn.*, **15**, 119.

⁵Contribution No. 408, from the Department of Botany and Plant Pathology, Kansas State College of Agriculture and Applied Science.

⁶Bates, J. C. 1891. A method for clearing leaves. *Amer. Nat.*, **65**, 288. (See p. 44 of this issue.)

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

SEIFRIED, O. Neuzeitliche Sammlung und Ordnung histologischer Schnitte. *Zts. wiss. Mikr.*, 56, 367-71. 1939.

A new filing system for microscopical preparations is described. It consists of card board units (length 14½ in., width 4 in., metal bound) that accomodate 10 slides. The lower two-thirds of the slides fit into paper pockets which afford protection to the preparations. A special cabinet is provided having a series of drawers 14½ X 18 in.—J. M. Thuringer.

PHOTOMICROGRAPHY

ABRAHAMSON, E. M. An inexpensive microphotographic camera. *Science*, 91, 510. 1940.

A "Univex" camera, model AF (price \$1.00) can be adapted for photomicrographical uses as follows: Set the shutter for "time" and open it. With nail and hammer strike a smart blow at the lens and shake out the chips. Draw out the wire frame used as a finder, and remove the horizontal section by bending at the angles till breakage occurs. Bend the two side pieces down along the front of the lens board, and curve slightly to receive the ocular of the microscope. Remove the back of the camera, connect to the microscope tube, and focus by means of a ground glass or tracing paper 33 X 45 mm. Introduce a role of film (price \$0.10) and take six exposures at varying times. At least one exposure will give a sharp negative; no rules can be given since the time of exposure depends on the illumination.—J. A. de Tomasi.

HELLWIG, C. ALEXANDER. Routine microfilming of tumor slides. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 95-9. 1940.

The following method is proposed for routine microfilming: A 12.5 cm. metal focusing tube is fitted to the eyepiece of the microscope by an adapter ring. A ground glass at the top allows centering of the light source and focusing the field. The camera, which is also 12.5 cm. long, consists of a Bantam back attached to a Bakelite bellows and is held on the eyepiece of the microscope by gravity. The lower end of the camera, made of heavy brass, is provided with a dark slide. The best light source is a 100 watt Spencer microscope lamp with an iris diaphragm and a holder for Wratten filters B and G. It is focused on the plane mirror 8" away. Eastman Panatomic film F 828, 28 X 42 mm., is used. The following procedure is recommended: Set the focusing tube on the eyepiece. Focus the margin of the iris diaphragm sharply on the center of the ground glass. Switch off the light. Replace the tube by the camera. Remove the dark slide and expose the film by switching the light on and off. Replace the dark slide and remove the film. Develop preferably with Champlin No. 16 developer at 68° F. for 9 min. Fix 15 min. and wash in running water. Remove droplets of water by a piece of wet cotton, and dry by a fan. Use a fixed focus enlarger to produce 2½ X 3" pictures on Kodabrom No. 4 paper or lantern slides. Develop in Eastman D-72.—G. H. Chapman.

McCLENDON, J. F. Inexpensive microphotographic records. *Science*, 92, 194. 1940.

The suggestion made (Abrahamson, *Science*, 91, 510, 1940) that the lens of a "Univex" camera be destroyed in order to obtain a focus in photomicrographical work is opposed by the author. If the camera is placed in the same position

as the eye after focusing the microscope, the camera will be in focus. To center it in position, a paper or tin tube may be applied to the front of the camera, and slid down over the eyepiece. The tube is secured with liquid solder, or any other quick-drying nitrocellulose adhesive. The time exposure is usually about 1 sec.—*J. A. de Tomasi.*

MICROTECHNIC IN GENERAL

HAMILTON, J. M. Isobutyl methacrylate as a mounting medium for histological preparations. *Science*, 92, 44. 1940.

Celloidin sections, 30×50 mm. and 25μ thick, of brain tissue mount unsatisfactorily in isobutyl methacrylate polymer (Du Pont Co.). This material in xylene solutions of various concentrations, when used without a cover glass, causes the sections to warp on hardening, and the resulting surface is rough. Under a cover glass, the sections crack and split. The thionin stain for Nissl cells remains unaffected, but the blue of the myelin sheaths stained with the Weil method turns a dull gray and some of the details are lost.—*J. A. de Tomasi.*

HOFFSTADT, RACHEL, and OMUNDSON, DOROTHY. A convenient apparatus for the manipulation of eggs in the study of the chorio-allantoic membrane. *Science*, 91, 459-60. 1940.

A search has been made for a convenient arrangement usable in high power microscopy for studying the virus of infectious myxomatosis and its action on the chorio-allantoic membranes of chick and duck embryos. As a result a simple apparatus is described which is composed of appliances available in any laboratory. After culturing the virus on the membrane, an opening of 2-3 cm. in diameter is made in the egg shell, and a wall of some 2 mm. is built around it with a mixture of equal parts of vaseline and paraffin. A few drops of saline are placed on the membrane, and a cover slip is sealed to the paraffin. Another small opening in the shell allows tight application by paraffin of a glass tube in correspondence with the egg air sac. By means of rubber tubing and a clamp, air can be blown gently into the air sac, and the level of the membrane raised or lowered according to need.—*J. A. de Tomasi.*

JENSEN, H. W. A speedier and less costly method of concentration in nitro-cellulose imbedding. *Science*, 91, 509-10. 1940.

Any laboratory using celloidin as an imbedding agent will soon accumulate amounts of it that can again be put to use in the following way: Redissolve in ether-alcohol and let settle for a few days. Decant into petri dishes to a depth of $\frac{1}{4}$ in. Let it solidify and cut into 1 in. squares. Gather pieces on a No. 1 pin, and dry completely. These pins may be used instead of the usual troughs attached to the inside of the cork in the imbedding bottle. Advantages: reduced cork leakage; concentrating of the 10% celloidin shortened by several days; for most cytological materials, celloidin stages cut down to 2, 6, 10% steps without apparent damage.—*J. A. de Tomasi.*

SUNTZEFF, V. and SMITH, IRENE. The use of plastic as a substitute for cover glasses. *Science*, 92, 17-8. 1940.

The shortage of cover glasses caused by the conflict in Europe has again suggested the use of plastic materials in their stead. The authors suggest as a suitable plastic "Vue Pak" (Monsanto Chemical Co.) which can be obtained in sheets 0.005 in. or 0.127 mm. thick, comparable to the thickness of No. 1 cover glasses. The refractive index is 1.49-1.50, very close to that of glass, and the resistance to heat is 140-180° F. Also, the effect of sunlight, weak acids and alkalies is negligible. Dried slides thus covered remain unchanged when repeatedly held at 110-115° F. for 12 hr., and cooled. The plastic sheet is cut to cover glass size, and kept between sheets of tissue paper; for cleaning, it may be dipped in 50% alcohol and dried immediately. As the slips have a tendency to curl, the drying should be carried out slowly at room temperature. By substituting isobutyl methacrylate polymer (Du Pont Co.) for Canada balsam as a mounting medium, drying does not require more than a day, and results in the whole are improved.—*J. A. de Tomasi.*

DYES AND THEIR BIOLOGICAL USES

CHALKLEY, L. Organic mercury derivatives of basic triphenylmethane dyes. *Science*, 91, 800. 1940.

Owing to the characteristics of the basic triphenylmethane dyes, development of organic mercury derivatives seems desirable and promising in such fields as bacteriology and pharmacology. It is found that derivatives can best be prepared by a two-stage process where a dye intermediate is first mercurated, and the resulting compound then converted into the dye. A small amount of dicyanomercury malachite green has thus been prepared, and is being offered freely to interested experimenters.—*J. A. de Tomasi*.

FORSTER, R. PH. A renal clearance analysis of phenol red elimination in the frog. *J. Cellular and Comp. Physiol.*, 16, 113-22. 1940.

Inulin clearance, a measure of glomerular filtration, was compared with simultaneous phenol red clearance to analyse the tubular secretion of the dye.

For phenol red concentrations in the plasma below 0.5 mg. per 100 ml., 97% of the total dye was eliminated by the kidney tubules. The maximal rate of tubular secretion, 5.0 mg. per kilogram body weight per hr., was attained at a plasma concentration of approximately 5 mg. free phenol red per 100 ml. The phenol red to inulin clearance ratio averaged 7.0 at a total phenol red concentration in the plasma of 1.0 mg. per 100 ml., and below 1.0 at a plasma concentration of 40 mg. phenol red per 100 ml.—*L. Farber*.

FOX, C. I. Is sulfanilamide bacteriostatic under anaerobic conditions? *Science*, 91, 477-8. 1940.

Methylene blue, introduced either initially or after full growth of *Escherichia coli*, proves that anaerobic, reducing conditions interfere with sulfanilamide bacteriostasis. The medium in which the organism is grown is that suggested by Quastel, Stephenson, and Whetham (*Bioch. J.*, 19, 304, 1925). In the simple lactate medium, where reducing conditions prevail, sulfanilamide has no effect upon growth; in the "anaerobic" lactate-nitrate medium in which the methylene blue is not reduced, the bacteriostatic action of sulfanilamide is evident. These findings may help to interpret the unsatisfactory effect of sulfanilamide therapy in mastoiditis and other closed infections which are thought to be accompanied by anaerobic glycolysis.—*J. A. de Tomasi*.

HÖBER, R., and BRISCOE-WOOLLEY, P. M. Further studies on conditions determining the selective renal secretion of dyestuffs. *J. Cellular and Comp. Physiol.*, 16, 63-70. 1940.

Sulfonic acid azo-dyes having a bilateral elongated molecule with the sulfonic acid groups on one half of the molecule are secreted by the proximal tubules of the excised and Ringer- or blood-perfused frog kidney. Triphenylmethane dyes are not secreted by the kidney perfused with Ringer solution, but some are secreted by the freshly excised kidney. Furthermore, perfusion with Ringer solution for 1 hour prior to excision inhibits or greatly diminishes the secretion of those triphenylmethane dyes previously secreted, but does not affect the secretion of the sulfonic acid azo-dyes. The authors conclude that two mechanisms are involved in the secretion of dyes, one depending on a physico-chemical affinity between dye and cell, the other involving some component in the cell environment which is not present in Ringer solution.—*L. Farber*.

ANIMAL MICROTECHNIC

CRAIG, JANE S. A simple stain for tissue cultures. *Science*, 92, 226. 1940.

In hanging drop tissue cultures in plasma, most stains suitable for the cytoplasm also stain the plasma of the clot. The following method, for brain tissue, reveals clearly the finest processes of the cells, nuclei, and certain cytoplasmic inclusions: Remove from the cover slip all paraffin and vaseline with cotton plectgets soaked in chloroform. Fix 24 hr. in 10% neutral formalin, or absolute alcohol. Stain 1 hr. in 1% aqueous toluidine blue, and wash in 2 changes of distilled water. Dehydrate 2-3 min. in 85%, 2-3 min. in 95% alcohol. Differentiate 5-10 min. in absolute alcohol under the microscope. Clear in xylene, and mount in balsam, or nevillite.—*J. A. de Tomasi*.

EINARSON, L., and BENTSON, K. *Bemerkungen zur progressiv-selektiven farberischen Darstellung der Nervenzellen in Paraffin- und Zelloidinschnitten.*
Zts. wiss. Mikr., 56, 265-72. 1939.

The authors emphasize the wide range of usefulness of the Galloxyanin stain for nervous tissues. Prepared according to the original formula, it stains only the Nissl substance, nuclear chromatin, nucleoli, and nuclei of glia cells.

Celloidin sections are stained by the following procedure: Dissolve 5 g. aluminum sulfate and 5 g. chromalum in 100 ml. of dist. water. Add 0.5 g. anthracene blue. Shake and bring to a boil for 10-15 min. (The reaction of this solution is about pH 1.95; but it is adjusted by means of buffers, as explained below.) Stain for 24-48 hr. Paraffin sections are stained for 48 hr. No differentiation is required and over-staining does not occur.

Lowering the pH from 2.09 to 1.20 produces selectivity which gradually eliminates every trace of co-staining any other but the specific substances. Conversely, raising the pH from 2.09 to 4.46 increases co-staining of glia cells and fibrillar elements, cytoplasm, and cell processes. At pH 2.60-3.80 histological pictures of exceptionally high quality are produced.

The buffering of the stain is given in a special table. For human cerebral-cortex stain buffered at pH 1.95-2.59 is used; for mesencephalon, pons, medulla oblongata, and cerebellum, pH 2.68-2.73 and for spinal cord pH 2.73-3.25. After staining, section is washed in distilled water, finished thru alcohol, carbolic acid, and balsam. This technic is based on formalin-fixed material, and is suitable both for paraffin and celloidin sections. Zenker-fixed material yields especially good cytological pictures; however, the unspecific co-staining is considerably modified.—J. M. Thuringer.

FOGG, L. C., and WARREN, S. *The centriole in radiated tumor tissue.*
Science, 91, 528-9. 1940.

Previous work on the cytology of Walker rat carcinoma 256 had not pointed out the presence of centrioles. X-ray irradiation of such material reveals paired bodies in the interkinetic stage of the cells of this tumor. The type and dosage of radiation is as follows: 200,000 volt X-ray; 50 cm. distance; filter 0.5 mm. Cu, 1 mm. Al; one dose 2400 r.; rate of irradiation, 40.6 r. per min. After 18, 48, 72, 96, and 120 hr. of irradiation, the animals are killed and the tissue fixed either in Bouin or Zenker. Staining is most satisfactory with: Heidenhain's iron hematoxylin and eosin, eosin Y and methylene blue, or phosphotungstic acid hematoxylin. A quantitative analysis of cells showing centrioles reveals that irradiation at various intervals detects a wide variation in the frequency of multiple centrioles. Their number increases up to 72 hr., then sharply decreases to a normal level after 120 hr. of treatment.—J. A. de Tomasi.

FOLEY, JAMES O. *A new silver method for staining nerve fibers in blocks of nervous tissue.* *Anat. Record*, 73, 465-72. 1939.

A silver method for staining peripheral nerves, designed to display the smallest axones distinct and separate, is described as follows: Fix the stretched nerve 24 hr. at 4° C. in the following solution: potassium bichromate (3-5% aqueous solution), 49 cc.; ammonium hydroxide (28%), 1 cc.; pyridine (concentrated), 15 cc.; ethyl alcohol (95%), 50 cc. Wash 15-30 min. in each of a series of alcohol-pyridine mixtures, composed of 15 vol. pyridine to 85 vol. dilute alcohol, the alcohol in the first bath being 50%, in the 2nd 40%, the 3rd 30%, the 4th 20% and the last 10%. (In the last solution no yellow color should be extracted from the tissue.) Pass thru an ascending series of aqueous pyridine (10%, 20%, 30%, 40%, 50%), 15-30 min. each. Remove tissue from stretcher, snip off knot at one end, and thread thru strips of cerebral cortex preserved in 50% pyridine. Pass thru an ascending series of aqueous pyridine (60%, 70%, 80%, 90%), at least 30 min. each; absolute pyridine 24 hr., 2 or 3 changes. Trim with razor 3-5 mm. in diameter. Run thru a descending aqueous pyridine series (from 90% to 10% in steps of 10%), 15-30 min. each. Wash 36 hr. in distilled water, changing every half hour during the working day. Leave 3-5 days in AgNO₃ at 37.5° C. Rinse few seconds in water. Treat 48 hr. with Ranson reducer (4% pyrogalllic acid in 5% formalin). Dehydrate in alcohols and imbed.

The author recommends his celloidin paraffin embedding for thin sections and cytological details. For 10-20 μ sections he recommends imbedding in butyl alcohol and paraffin. Some sections should be toned and counterstained under the microscope.

A method quite similar to the above is given for central nervous system tissues. Such tissues are perfused 1-2 hr. with the fixing agent and are then placed in fresh fixative in the ice box for 48 hr. The silvering is increased to 5-7 days. Embedding in butyl alcohol and paraffin is recommended. The author states that celloidin or dioxan will tend to extract the silver. Sections may be toned and counterstained for various studies.—*S. I. Kornhauser.*

GRAFFLIN, ALLAN L. Histology of the thyroid and parathyroid glands in the mountain gorilla, with observations upon autofluorescence, fat and pigment. *J. Morphol. & Physiol.*, 67, 455-70. 1940.

Tissues were fixed in 5% formalin, in which they remained for about 7 months before this study was made. Frozen sections were mounted unstained in C.P. glycerin and studied under both the ordinary and the fluorescent microscopes. Sections for the study of fat were stained either for 5 min. with a saturated solution of Sudan III in 70% alcohol containing 1% KOH, or for 24 hr. in a saturated solution of the dye in 40% alcohol. Routine histological preparations were made by embedding tissues in paraffin, via absolute alcohol and chloroform, serially sectioned at from 5 to 10 μ , and stained with hematoxylin and eosin.—*Elbert C. Cole.*

HESS, M. The localization of acid azo dyes in tumours. *J. Path. & Bact.*, 51, 309-11. 1940.

Necrotic changes in two transplantable carcinomas of mice and rats were clearly demonstrated after the intravenous injection of Chicago blue 6B (source not stated). For mice, 15 mg. of dye in 0.5 cc. of distilled water served as the dose, and for rats, 60 mg. in 1.5 cc. This was followed by fixation in Susa for 2 hr., dehydration in alcohol not longer than 5 hr., and 2 changes of chloroform. Paraffin sections were treated with iodine and examined both unstained and after light staining with carmalum.

After 24 hr., necropsy revealed diffuse blue in most organs and tissues, but brain, lungs and spleen appeared dye-free. Carcinoma cells were unstained, but fibroblasts and stroma cells in the tumor were laden. Degenerating cells were diffusely stained. Mesodermal elements thruout the body stored large amounts of dye.—*S. H. Hutner.*

KRAJIAN, ARAM A. Frozen section method for preparation of permanent tissue sections. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 82-8. 1940.

Frozen sections may be made in place of paraffin or celloidin sections if the following suggestions are followed: The microtome knife should have a shorter bevel on the under side. In cutting sections, skin, capsule or other covering must face the knife. Put a drop of water on the microtome. Press the block firmly on it and freeze slowly and evenly until about one-half of the block is frozen. Cut off the unfrozen portion and shave. (If the tissue is too cold, it will be hard and white. If too soft, it will disintegrate in water. If it is cut too fast, the sections will curl or break into shreds. Firmer tissues give thinner sections for the same setting of the microtome.) Soak loose texture tissues in a Stender dish containing fresh serum. Cover any pieces that float with cotton. Put the dish on top of the paraffin oven overnight. Without washing cover the block with dioxan 3-5 hr. For fatty tissues cut sections 15 μ , leave them on the edge of the knife, apply 2-3 drops of 95% ethanol 1 min., and transfer them to water by the tip of the little finger. If this does not make the sections spread, keep them in the ethanol 30-45 min. and then in water. If this does not help, put the block in equal parts of acetone and 95% ethanol, fix again in formalin and then cut. After affixing sections to the slide, drain 15-60 sec. but do not let them become dry. Hold the slide at a slight angle, add 2 drops of anhydrous isopropanol (Union Carbide and Carbon Company) or absolute ethanol. Blow until the section is white and then blot with Whatman filter paper No. 2. Do not blot mucoid tissues. Flood the section 1-10 min.

with isopropanol. Drain carefully, blot, and dip rapidly twice in the following solution: equal parts absolute ethanol and ether, 88 ml.; thick celloidin, 7 ml.; gum mastic, 1 ml. (gum mastic, 25 g.; 95% or absolute ethanol, 85 ml.; stand 1 week). Blow gently. Stain immediately in alum hematoxylin, 3-5 min. Rinse in tap water until blue (1-2 min.). Destain in the following solution: HCl, 1 ml.; 70% ethanol, 100 ml. until no more color runs. Rinse in tap water. Dip once or twice in 2% ammonia water until blue. Dip in tap water. Wipe the slide but not the tissue. Put the slide in 95% ethanol or 88% isopropanol 1-10 min. Add 2-3 drops of anhydrous ethanol or isopropanol. Repeat this addition twice. Dip several times in eosin until the background is stained evenly red (10-30 sec.).

The eosin stain is prepared as follows: aq. eosin, 5 g.; dist. water, 10 ml.; glacial acetic acid, 10 ml.; conc. HCl, 2 ml. Mix with a glass rod. Put the mixture in a 56° C. oven 12-16 hr. Dissolve the mass in 10 ml. absolute ethanol or anhydrous isopropanol and 20 ml. acetone. Stir. Keep 5 hr. at 56° C. Let settle. Remove the clear supernatant and add it to 1,500 ml. of the following: phenol crystals, 1 part; neutral xylene, 3 parts. Use the clear portion of this eosin solution. (For standardization see *Arch. Path.*, 25, 376-7, 1938.)

Put the stained slide in carbol-xylol 3-4 min. and drain completely. Put in 3 changes of xylol, 2 min. each, draining each time. Mount in neutral gum dammar (Eastman Kodak company, saturated solution in neutral xylol). If sections should become detached from the slide during the staining process, flood the section on the slide with isopropanol 2-5 min., drain, blot, and dip twice in the celloidin solution.—*G. H. Chapman.*

MORRISON, MAURICE, and SAMWICK, A. A. Restoration of overstained Wright films and a new method of staining blood smears. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 92-3. 1940.

Poorly stained blood films may be corrected by flooding with Wright's stain for 15-30 sec. and rinsing with distilled water. Fresh films may be more easily stained by the following technic: Cover with Wright's stain and allow to evaporate. Add more Wright's stain, 15-30 sec. Wash with distilled water.—*G. H. Chapman.*

SILLS, BERNARD. A simple aid in making a blood smear. *J. Lab. and Clin. Med.*, 25, 1802. 1940.

The following device is described which overcomes many of the difficulties of the Ehrlich two-cover glass method: Place two perfectly clean cover glasses face to face. Gently warm the tip of a waxed stick (the Mexican wax match is satisfactory), place it in contact with the edge of the top cover glass and allow it to harden. Lift the top cover glass with the stick as a handle, touch it to the drop of blood secured in the usual manner, and place it on the lower cover slip so that none of the corners coincide. As soon as the drop spreads, pull the cover glasses apart by grasping the lower one by the corners and the upper one by the stick. The glasses need not be touched with the fingers until after the drop is spread.—*John T. Myers.*

PLANT MICROTECHNIC

BATES, JAMES C. A method for clearing leaves. *Amer. Nat.*, 65, 288. 1931.

The following method has proved quite effective for clearing and decolorizing whole leaves darkened by preservatives and containing considerable quantities of tannin.

The leaves to be cleared are placed in a test tube and covered with a saturated solution of chloral hydrate for 48 hr. or longer. The chloral hydrate is then poured from the material and enough $KClO_3$ added to fill the bottom of the test tube to about $\frac{1}{2}$ in. The material is then covered with concentrated HNO_3 and let stand until the leaves begin to change color. From 10-30 min. is usually sufficient. The HNO_3 is then poured out, leaving the $KClO_3$ and leaves in the test tube, and the material again covered with a saturated solution of chloral hydrate. In this solution there will be a continuous liberation of chlorine gas and within less than a week the leaves should be quite transparent. If necessary the HNO_3 treatment may be repeated.—*Reprinted from original.*

CHRISTENSON, B. V., and STOKES, R. C. A pharmacognostical study of *Serenoa serrulata* (saw palmetto). *J. Amer. Pharm. Assoc.*, 29, 199-206. 1940.

On account of the presence of extremely thick cell walls and numerous lignified stone cells in the fruit of the saw palmetto, it was necessary to devise special methods of treatment for rendering it suitable for sectioning. The seed was removed after making a transverse incision around the middle portion of the fruit and lifting off one-half. The hard seed was boiled in water for 24 hr., then placed in a 25% solution of HF for a week, thoroly washed in water and left for a week longer in equal parts of 95% alcohol and glycerin. The pericarp was steamed on a water bath for 2 hr. and cut into longitudinal sections. Paraffin sections 15 μ thick were satisfactory for histological study.—*Merritt N. Pope*.

MCLEAN, ROBERT S., and IRELAND, EDWARD J. Rapid staining methods in plant histology. *J. Amer. Pharm. Assoc.*, 29, 318-21. 1940.

Rapidity of staining was accomplished by igniting and burning off, on the slide, the solvent of a small amount of a stain dissolved in a high grade of alcohol. The stains, American made, were generally 1% in 95% alcohol except in the case of acid fuchsin and orange I, where 0.5% was used. Methyl blue, due to low solubility in 95% alcohol, was dissolved in 80% alcohol. When the solubility of a stain was less than 1%, a saturated solution was used. No apparent damage to cells or contents was observed in more than 200 slides stained. The following procedure was followed: Place 2-4 drops of primary stain on slide from absolute alcohol. Ignite and allow solvent to burn off completely. Wash briefly but thoroly in 95% alcohol. Place 4-6 drops of counterstain on slide for 10-20 sec. With woody sections or with stains acting slowly, such as methyl blue and orange G, burn off as with primary stain. Wash in 95% alcohol, clear and examine. To insure proper differentiation, use a 5-10 sec. staining period, with washing and examination repeated until adequately stained, or if sections are 5 mm. or more in length, hold over a white surface until color of section changes to that of the counterstain.

Best combinations for leaf sections proved to be methylene blue with eosin or erythrosin; Victoria green with picric acid; safranin A or rhodamine B with methyl blue. For stem and root sections the best were methylene blue with eosin, erythrosin or orange G; malachite green with acid fuchsin or orange I; and safranin A with methyl blue. A shorter method resulted from using a combination of stains and counterstains in a single solution, burned off as in the previous method. Washing time, generally 5-15 sec., varied with stain combinations and nature of sections. Also such double stains for blood, as Wright's and Giemsa's standardized solutions and Wright's and Jenner's saturated solutions in 95% alcohol, proved satisfactory after ignition. Giemsa's stain should be stopped, i.e., flame blown out, when the glycerin begins to bubble. The following combinations proved satisfactory (numbers following stains indicate volumes in combination): Malachite green, 1, or Victoria green, 1, with acid fuchsin, 10; basic fuchsin, 1, with methyl blue, 10; safranin A, 3, with orange G, 20; methylene blue, 2, with acid fuchsin, 10; methylene blue, 3, with erythrosin, 20. Where cork was present in the section, a triple color combination was obtained with the blood stains and with methylene blue in combination with acid fuchsin, orange G or erythrosin.—*Merritt N. Pope*.

MICROORGANISMS

BRYAN, C. S., DEVEREUX, E. D., HIRSCHY, W. C., and CORBETT, A. C. The use of brilliant green, sodium azide and dextrose in the microscopic and Hotis tests for streptococcal mastitis. *North American Veterinarian*, 20, 41-6. 1939.

Experiments were carried out to determine the effectiveness of brilliant green and sodium azide in suppressing bacteria other than streptococci and to determine the value of dextrose and physical factors in obtaining maximum growth of streptococci during the time interval of the microscopic and Hotis tests. (Hotis and Miller, U. S. Dept. Agr. Circ., No. 400, 1936.) From tests on the milk of several hundred cows it was concluded that better results were obtained from a selective preservative containing brilliant green, sodium azide and dextrose than from either preservative used alone and the use of a selective preserva-

tive yielding a final dilution of 1:50,000 of brilliant green, 1:15,000 of sodium azide and 1:1000 of dextrose in the milk is recommended. This preservative increased the accuracy of the microscopic test as compared to the use of brilliant green alone and greatly reduced the time required to read the results by suppressing both the udder micrococci and contaminating bacteria. The use of this preservative in the Hotis test increased the 24-hr. reading from 60 to 81% efficiency and reduced the number of negative cows giving positive tests from 7.8 to 2.6%. Similar improvement was obtained in the 48-hr. reading. The addition of dextrose compensated for the low lactose content of the milk of mastitis-infected cows and permitted better growth of the streptococci in the sample.—*Colin W. Whittaker.* (*Cited from Chem. Abstr., 34, 1850. 1940.*)

CASTRO, GUILLERMO. The rapid diagnosis of malaria from thick blood smears. *J. Lab. & Clin. Med.*, 25, 1808-10. 1940.

The following modification of the Barber and Kemp method (Pub. Health Rep., 44, 2330, 1929) is given: Put a drop of blood about 3 times the size necessary for a thin smear on a slide and spread in a circle about $\frac{1}{2}$ inch in diameter made by a wax pencil. Place the slide on a hot plate at 50°-60° C. where it will dry in about one minute. Stain for 3 to 5 min., using a mixture of 3 drops of distilled water and 1 or 2 drops of Giemsa's stain. Wash carefully in a tray of distilled water and dry on a hot plate in an inclined position. The method is rapid, the red cells are completely decolorized, the parasites are deeply and distinctly stained and any basophilic stippling is clearly demonstrated.—*John T. Myers.*

GOULD, S. E. Incidence of trichinosis among county hospital patients in the Detroit area. *Amer. J. Clin. Path.*, 10, 431-59. 1940.

The following two methods are recommended for searching for larvae of *Trichinella spiralis*: (1) Press pectoral muscle and diaphragm between two pieces of plate glass in a metal frame compressed by set screws and examine under a dissecting microscope. (2) Digest these tissues in artificial gastric juice (1% pepsin and 0.7% HCl) 15-24 hr. at 37° C.; allow the chilled digest to settle and wash the sediment. Examine with the microscope. The first method gave 15.4%, the second 17.1%, and a combination of the two 20% positives.—*G. H. Chapman.*

KELLY, CORNELIUS B. Brilliant green lactose bile and the standard methods completed test in isolation of coliform organisms. A comparative study. *Amer. J. Pub. Health*, 30, 1034-9. 1940.

This study was made to determine the efficiency of brilliant green lactose bile broth (Standard Methods formula) as a confirmatory medium for coliform organisms, particularly from shellfish and shellfish-bearing waters. Parallel inoculations were made into this medium and onto EMB agar from all lactose broth tubes which were submitted to the completed test, and results from the two series compared. Comparative tests on 2,087 water samples, of which about 90% were sea waters, indicated that the presence of gas in brilliant green lactose bile broth is a more accurate criterion of the presence of coliforms than the Standard Methods completed test using solid confirmatory media alone. Similar results, but not so marked, were obtained on 2,347 shellfish samples. Definite seasonal variation was observed. Considering all samples, gas production in brilliant green lactose bile broth was obtained with 101.2% of the total coliforms isolated by both methods, while the Standard Methods completed test recovered 93.8%.—*M. W. Jennison.*

NABEL, K. Über die Membran niederer Pilze besonders von *Rhizidiomyces bivellatus* nov. spec. *Arch. Mikrob.*, 10, 515-41. 1939.

The method of Van Wisselingh for identifying chitin and cellulose is adapted for use with membranes of the lower molds by destroying by heat nearly all the other substances present. The procedure is as follows: In a glass tube 6 cm. \times 6-7 mm., sealed at one end, place a layer of glycerin 1-2 cm. thick. Introduce the object to be studied into the glycerin, seal off the open end and heat to 900° C. in

an oil bath. Cool, cut off the tube close to the surface of the glycerin, harden the greatly softened tissue in abs. alcohol and wash in dist. water. Transfer tissue to another glass tube containing conc. KOH, seal off and reheat in the oil bath, this time to 180° C.; cool, open, harden in alcohol and wash in dist. water. Bring the material onto a slide and add picric acid. A permanent yellow color indicates chitin while cellulose is shown if the color can be washed out. Add a few drops of I-KI solution and 1% H₂SO₄. Chitosan is shown by a red-violet coloration (Ostwald's color tables No. 10, first part between ga and ia). Treat now with conc. H₂SO₄ and the red-violet color disappears and the chitosan dissolves. If there is no chitin present in the membrane, the tissue remains uncolored after the treatment with I-KI solution and 1% H₂SO₄, but on addition of the conc. H₂SO₄ the presence of cellulose is shown by a clear blue coloration (Ostwald's color table No. 13, first U blue, between ga-ia-la).—*Merritt N. Pope.*

ROE, M. A., LILLIE, R. D., and WILCOX, A. American azures in the preparation of satisfactory Giemsa stains for malaria parasites. *Public Health Reports*, 55, 1272. 1940.

Using a modified Nocht technic, the authors have explored the thiazin dye group in combination with eosin for dyes and proportions giving satisfactory staining of malaria parasites in thin and thick blood films. Methylene blue stains parasite cytoplasm satisfactorily but fails to demonstrate parasite chromatin. Azure B gives clear, sharply defined, purplish red chromatin and sharply defined cytoplasm, a somewhat lighter blue than with methylene blue. Azure A gives denser, deeper reddish purple and less sharply defined chromatin and a grayish lavender parasite cytoplasm which contrasts poorly with red corpuscles or with the background of thick films. The most satisfactory staining was given by mixtures of azure B (1 part) and methylene blue (1 to 4 parts) with the appropriate amount of eosin and with or without a small amount of azure A (0.2 part). Three of these formulac were made up of corresponding quantities of the dry dyes in glycerin and methyl alcohol and gave very satisfactory results when a 1:50 dilution at pH 7.0 for 45 min. was used.

The formulae, in parts by weight, are: (A) Azure A 0.5, azure B 2.0, methylene blue 2.7, eosin Y 5.0, in CP glycerin 50 and CP methyl alcohol 50. (B) Azure B 2.5, methylene blue 2.7, eosin Y 5.0 in the same solvent. (C) Azure B 1.7, methylene blue 3.4, eosin Y 4.9 in the same solvent. These weights are in terms of pure dye and corrections should be made for dye content.

Of these formulae, some observers prefer A, some C. The results are closely similar to those obtained with Grubler's Giemsa solution.—*R. D. Lillie.*

ROGERS, T. HOWARD. The inhibition of sulphate-reducing bacteria by dyestuffs. *J. Soc. Chem. Ind.*, 59, 34-9. 1940.

Bacteria which produce H₂S from inorganic sulfates are of economic importance because of the damage done by the H₂S to materials with which it comes in contact, e.g. corrosion of metals in various industrial conditions. Inhibition of these organisms is often impossible by the use of ordinary disinfectants because they may have corrosive properties. The development of sulfate-reducing bacteria may be inhibited by the use of dyestuffs derived from 3:6-diamino-acridine, such as acriflavine, proflavine, or No. 914 (a flavine dye produced by Imperial Chemical Industries). These dyes are non-corrosive, and are effective in very small quantities. The concentrations of dye required are a function of the degree of contamination; but, as far as it is possible to ascertain from laboratory tests alone, the concentration of dye required in most practical conditions appears to be 1 in 250,000 or less.—*A. P. Bradshaw.*

SCHAEDE, R. Zum Problem des Vorkommens von chromatischer Substanz bei Bacterien und Actinomycetes. *Arch. Mikrob.*, 10, 473-507. 1939.

Study of the chromatic substance of bacteria and Actinomycetes by means of the Fuelgen reaction is reported in detail. Endophytes are fixed in the tissue of the host plant using the fluid of Juel generally (but also Benda's and sublimate in glacial acetic acid) embedded in paraffin and sectioned 5 μ thick. A 6-minute hydrolysis for material fixed in Juel's fluid is satisfactory. A new method for

free-living organisms, which avoids drying on the slide and consequent injury to cell contents, is described as follows: Prepare fixative by adding enough water to dried egg white to make a thick flowing mixture which will "thread" when dropped from the stirring rod. Use camphor as a preservative. In making the preparation mix a small mass of the culture with a drop of 0.8% NaCl solution on a slide. Carry over to a clean slide enough of the mixture for a streak preparation and invert for 2 min. over a dish of an osmotic acid fixing fluid containing no acetic acid. Place a small drop of albumin fixative on the slide near the drop of fixed bacteria; mix quickly with the inoculating needle and streak broadly. Before drying can take place, put the slide in 50% alcohol for 10 min. Wash with several drops of 70% alcohol and leave in 95% alcohol for at least 24 hr. Treat by the method of Fuelgen, hydrolizing 8 min.—*Merritt N. Pope.*

SOYANO, Y. A new technique: "mica method" for vital observation. (In Japanese with English resume). *Botany and Zoology: Theoretical and Applied* (Tokyo), 8, 106. 1940.

To overcome the difficulty of focusing thru the depth of a drop in the case of hanging-drop preparations, the author touches a clear mica flake to the drop. Because the flake is very light, surface tension holds it close beneath the cover slip, spreading the drop thin and permitting ready focusing.—*J. L. Mohr.*

STAIN TECHNOLOGY

VOLUME 16

APRIL, 1941

NUMBER 2

THE PREPARATION OF STEM SECTIONS OF WOODY HERBARIUM SPECIMENS

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ABSTRACT.—A procedure is described for sectioning the stems of woody herbarium specimens by the paraffin method. The specimens are cut into convenient sizes; boiled for one-half to one hour in order to exclude air and soak up the cell walls and membranes; cooled and placed in 5% NaOH for 24 hours in order to expand the collapsed cells and remove excessive coloring matter; washed in running water for a few hours; placed in hydrofluoric acid until sufficiently softened to cut easily with a razor blade; washed in running water for 24 hours to remove the acid; dehydrated and embedded in paraffin of high melting point (56°-58° C.) according to the n-butyl alcohol method; sectioned with the rotary microtome and completed by the ordinary method. Soaking the paraffin blocks in water for a period of several hours to a day or more before sectioning greatly improves the cutting and reduces electrification of the paraffin ribbon. The method proves satisfactory not only for herbarium material but for seeds and specimens of old bark and wood varying in hardness from balsa to ebony. For seeds or specimens containing only xylem, the NaOH treatment should be omitted.

In determining the nodal condition of plants and in dealing with various other problems connected with stem anatomy, it frequently becomes necessary to resort to herbarium specimens for materials. The drastic treatment which the specimen undergoes during pressing and drying often results in the collapse of cells and crushing of the softer outer portions of the stem. By soaking or boiling the stems in water one may be able to get satisfactory sections of the woody portions, but the softer parts usually fall away or become badly torn during the sectioning process. Embedding in celloidin is the pro-

¹The work reported in this paper was done at the Biological Laboratories, Harvard University, Cambridge, Mass.

cedure usually followed when complete sections are desired. Since this method is long and rather costly the writer has attempted to apply the *n*-butyl alcohol technic², making such adjustments as appeared necessary.

The first problem is to cut the dried herbarium specimen into convenient sizes. This may usually be done best with a sharp knife or razor blade while the specimen is dry, but if the bark is loose and flaky it may cut better after soaking a while in water. Care should be taken at this stage that the cells are not crushed or they will fall away and be lost.

Another problem is the restoration of the cells of specimens to an approximation of their normal shape and size. Since the flattened soft portions of the stem of pressed herbarium specimens are usually without excessive tissue distortion, it would seem that this restoration might be accomplished by means of a proper reagent. Considerable success is attained by treatment with NaOH and KOH. The range of concentrations most desirable is between 1 and 10%. Not only are the collapsed cells expanded but also much of the dark coloring matter commonly occurring in herbarium specimens is dissolved out. Staining is usually greatly improved by the clearing action of these reagents.

The next problem is the softening of the material prior to infiltrating with paraffin. A certain amount of softening action results from treatment with the above reagents but it is insufficient to soften hard materials. If the material contains an excessive amount of crystals or hard fibrous material a treatment with HF is necessary. The length of time required for the softening process will, of course, depend upon the nature of the specimen and the strength of the acid used. When the material cuts readily with a razor blade it should be removed from the acid. It is well to put an extra specimen in the acid to use as a test piece in order to avoid crushing the specimens intended for study.

The procedure from this point is that of the *n*-butyl alcohol method. (The celloidin technic may be employed here for those who favor this method and desire to use the sliding microtome.)

Another point which has to do with sectioning of especially difficult material is worth mentioning. It is well known that the cutting of paraffin-embedded material is often greatly improved by simply soaking the paraffin blocks in water for several hours or even for much longer periods. Certain materials, however, if left in water for

²Zirkle, Conway. 1930. The use of *n*-butyl alcohol in dehydrating woody tissue for paraffin embedding. Science, n. s., 71, 103-4.

more than a day will swell and crack badly. Material which cuts poorly may cut well after this treatment. Furthermore, it is often found that this procedure greatly reduces the electrification of the paraffin ribbon. If the water treatment fails to soften the specimens, the following method may often be resorted to with success: simply trim the paraffin blocks down until the specimen is exposed and place the specimen (exposed side down) in a waxed jar of strong HF (40-52%) for a day or more; remove from the acid and wash in running water for a period of at least several hours before cutting. Thin sections of old corn stems, hardwood twigs and seeds may be cut by this method. The chief advantage of this technic is that it will apply to material which is already embedded in paraffin and which might otherwise be discarded as being impossible to cut. In general, however, it is better to soften the material sufficiently before embedding in paraffin, because otherwise sections of specimens with heterogeneous structure or with gelatinous fibers may crack badly upon being affixed to the slides and allowed to dry previous to the removal of paraffin.

Following is a tentative schedule for preparing sections of herbarium material:

1. Cut the specimens into pieces of convenient sizes, selecting only the portions needed for study. If the outer portions of the stem break or crumple, try cutting after the next step.
2. Boil for one-half to one and a half hours in order to exclude air and soak up the cell walls and membranes. If the material is especially soft and fragile a treatment with cold water may suffice.
3. Cool and place in 5% NaOH for 24 hours in order to expand the collapsed cells and remove excessive coloring matter.
4. Wash in running water for a few hours. (Prolonged washing at this point is probably unnecessary because no insoluble salts should be formed with the acid of step 5.)
5. Soften in strong (40-52%) HF until the specimen can be cut easily and smoothly with a razor blade.
6. Wash in running water for 24 hours to remove the acid.
7. Dehydrate and embed in paraffin of high melting point (preferably 56°-58° C.) according to the *n*-butyl alcohol method.
8. Trim the paraffin blocks and soak in water for at least a few hours before sectioning with the rotary microtome.
9. If the cutting is still difficult place the paraffin blocks in HF for a day or more. Remove and wash well in running water overnight or longer before sectioning. (Step 9 should not be necessary and in fact may not prove satisfactory in all cases.)

10. Proceed from this point according to the ordinary method.

The above procedure has been tried with considerable success on soft, light woods (*Ochroma*) and such hard materials as: *Nothofagus*, *Quercus*, *Grubbia*, *Octoknema*, and *Cneorum* twigs; *Fraxinus*, old wood and bark; and dried heartwood of *Diospyros ebenum*.

For specimens containing only xylem, steps 1-4 may be omitted. Simply boil the blocks and proceed with step 5.

It appears that it should be possible to cut any plant material in paraffin by the above method.

STAINING PARAFFIN SECTIONS WITH PROTARGOL

6. IMPREGNATION AND DIFFERENTIATION OF NERVE FIBERS IN ADRENAL GLANDS OF MAMMALS

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ABSTRACT.—A series of experiments with protargol staining of nerve fibers in mammalian adrenal glands has yielded the following procedure: Fix 1-2 days in a mixture of formamide (Eastman Kodak Company) 10 cc., chloral hydrate 5 g., and 50% ethyl alcohol 90 cc. Wash, dehydrate and embed in paraffin. Cut sections about 15 μ and mount on slides. Remove the paraffin and run down to distilled water. Mordant 1-2 days in a 1% aqueous solution of thallous (or lead) nitrate at 56-60°C. Wash thru several changes of distilled water and impregnate in 1% aqueous protargol (Winthrop Chemical Company) at 37-40°C. for 1 to 2 days. Rinse quickly in distilled water and differentiate 7-15 seconds in a 0.1% aqueous solution of oxalic acid. Rinse thru several changes of distilled water for a total time of 0.5 to 1.0 min. Reduce 3-5 min. in Bodian's reducer: hydroquinone 1 g., sodium sulfite 5 g., distilled water 100 cc. Wash in running water 3-5 min. and tone 5-10 min. in a 0.2% gold chloride solution. Wash 0.5 min. or more and reduce in a 2% oxalic acid solution to which has been added strong formalin, 1 cc. per 100. (Caution. This last reduction is critical and over-reduction can spoil an otherwise good stain; 15-30 seconds usually suffices, and the sections should show only the beginning of darkening to a purplish or gray color.) Wash, fix in hypo, wash, dehydrate and cover.

During a study of the innervation of the adrenal gland, we have had an opportunity to experiment with its staining by Bodian's copper-protargol method (1937), by the 2-hour method (Davenport, McArthur and Bruesch, 1939) and by a number of modifications of impregnation, differentiation and reduction based on these methods. Differentiation of protargol stains by treatment with dilute acid before reduction of the silver was mentioned by Young (1939). In such differentiation, protargol acts like a basic dye, and in our hands proved to be a very effective means of destaining the glandular background. Since the step seemed to be important, we tried several organic acids in varying concentrations to determine the optimum type of treatment. Other experiments were made on the effect of adding copper to the protargol staining solution after the tissue had

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²Contribution No. 329.

been in it for 1 day. This "delayed copper" procedure combined with acid differentiation has given better stains than those obtained by earlier methods. Mordanting sections with nitrates before impregnating with protargol was tried also to observe differences between the effect of silver nitrate and nitrates of other heavy metals.

EXPERIMENTAL

1. *Source of tissue and method of fixation.* Adrenal glands from the following mammals were used: monkey, dog, cat, rat, guinea pig, mouse, cow and man. All fixation was for 1-2 days in formamide (Eastman Kodak Co.) 10 cc., chloral hydrate 5g., and 50% ethyl alcohol 90 cc. This fixative was decided upon as a result of the experiments made on fixation by Bank and Davenport (1940). It has given consistent results in the staining of nerve fibers and yielded tissue blocks which cut easily. Embedding in paraffin, cutting (15μ sections) and mounting were done in the customary manner.

2. *Impregnation.* The following variations were tried:

- a. Protargol 0.5 and 1% solution for 24 and 48 hr. at 37° C.
- b. Protargol as in a, 24 hr., then 10 cm. of 20-gauge copper wire wound or folded to fit the bottom of a Coplin jar dropped in and the impregnation continued for another 24 hr.
- c. Treatment with 1% solutions of nitrates of the following metals for 24 and 48 hr. at 60° C.: cadmium, copper, lead, silver, thallium (monovalent), and uranium. This "mordanting" was followed by washing and then impregnating 48 hr. at 37° C. in protargol solution.
- d. Protargol plus copper for 48 hr. at 37° C. as in Bodian's method.
- e. Silver nitrate solution for 24 hr.; then copper-protargol for 24 hr.

3. *Acid differentiation.* Oxalic acid was used in concentrations of 0.05, 0.1, 0.2, 0.5 and 1% aqueous solution and the slides treated for various periods of time from 5 seconds to 1 minute. Fumaric, formic, acetic, lactic, benzoic and hydrochloric acids were used in 0.1% concentration for 5 to 15-second periods of treatment.

4. *Reduction.* Amidol, bromhydroquinone, and hydroquinone as 1% solutions in 5% sodium sulfite were tried and, in the case of amidol and hydroquinone, a few sections were reduced with solutions to which 2 cc. and 4 cc. of glacial acetic acid were added to each 100 cc.

5. *Subsequent processing.* In the usual manner sections were washed thoroly after reduction, toned in 0.2% gold chloride, washed again and treated with 2% oxalic acid in 1% aqueous formalin solution. A final fixing in hypo, washing, dehydrating and covering in balsam completed the process. It was found that the reduction in formalin-oxalic solution was highly critical and care had to be taken that this step was not overdone (usually not over 15 to 30 seconds)

RESULTS

Impregnations as listed under *2a*, *b* and *d* above were successful. The value of the various metallic nitrates used as "mordants" (*2c*) came out in the following order: thallium (monovalent), lead, cadmium, copper, uranium, and silver. Thallium and lead nitrates improved the impregnation and differentiation of nerve fibers. Cadmium nitrate did no harm but was little or no better than a distilled water control or plain protargol without pretreatment. The last three, copper, uranium, and silver nitrates, gave poor stains of nerve fibers. Silver nitrate followed by copper-protargol (*2e*) failed. In comparing *a*, *b* and *d* it was evident that copper acted as a differentiating agent by suppressing the staining of connective tissue and enhancing cell nuclei and nerve fibers. The effect of copper was not the same as the acid differentiation and its use tended to nullify the subsequent effect of the acid. Acid differentiation without the use of copper destained cell nuclei. Addition of copper after the impregnation in plain protargol had been in progress for a day was an effective means of modifying its action, but sections so treated required acid differentiation (fig. 3).

The optimum concentration of oxalic acid used for differentiating after impregnation was found to be 0.05 to 0.1%. The weaker organic acids worked more slowly in 0.1% concentration but gave final results similar to oxalic acid. Hydrochloric acid did not differentiate, presumably because silver chloride is very insoluble. The time needed for actual immersion of the slides in 0.1% oxalic acid varied from 5 to 20 seconds. The optimum had to be determined by trial for the various tissue blocks, but was usually about 10 seconds. Washing for 0.5 to 1.0 minute thereafter thru two or three changes of distilled water helped to prevent surface precipitates. The time of washing was not critical but apparently should not be over 1 minute.

Amidol-sulfite solutions were quite unsuited for reduction because they gave a general stain without much differentiation of nerve fibers. Bromohydroquinone gave results similar to hydroquinone altho it was somewhat more vigorous in action and the final stains tended to be darker. Hydroquinone as used in Bodian's method was the most satisfactory reducer. The addition of acid to the reducing solution was not advantageous in amidol solutions and it caused complete failure with hydroquinone. Therefore, acidification of the reducing solution was not a substitute for the acid treatment before reduction.

DISCUSSION AND SUMMARY

On the basis of the foregoing experiments we are more than ever impressed by the uncertainty of applying to one type of tissue a

Plate 1. Photomicrographs which illustrate the staining of nerve fibers in the adrenal gland.

Figs. 1, 2 and 3. Sympathetic ganglia of the adrenal capsule of dog, stained as follows: 1. protargol, hydroquinone; 2. protargol, oxalic acid differentiation, hydroquinone; 3. protargol, copper-protargol, fumaric acid, hydroquinone. All $\times 500$. Figs. 2 and 3 show the improvement effected by acid differentiation.

Fig. 4. Nerve fascicle at the cortico-medullary junction, dog; silver nitrate, protargol, oxalic acid, hydroquinone. The very pale background and reduction of fiber staining intensity resulted from the silver nitrate treatment.

Fig. 5. A similarly located fascicle in monkey; protargol, oxalic acid, hydroquinone.

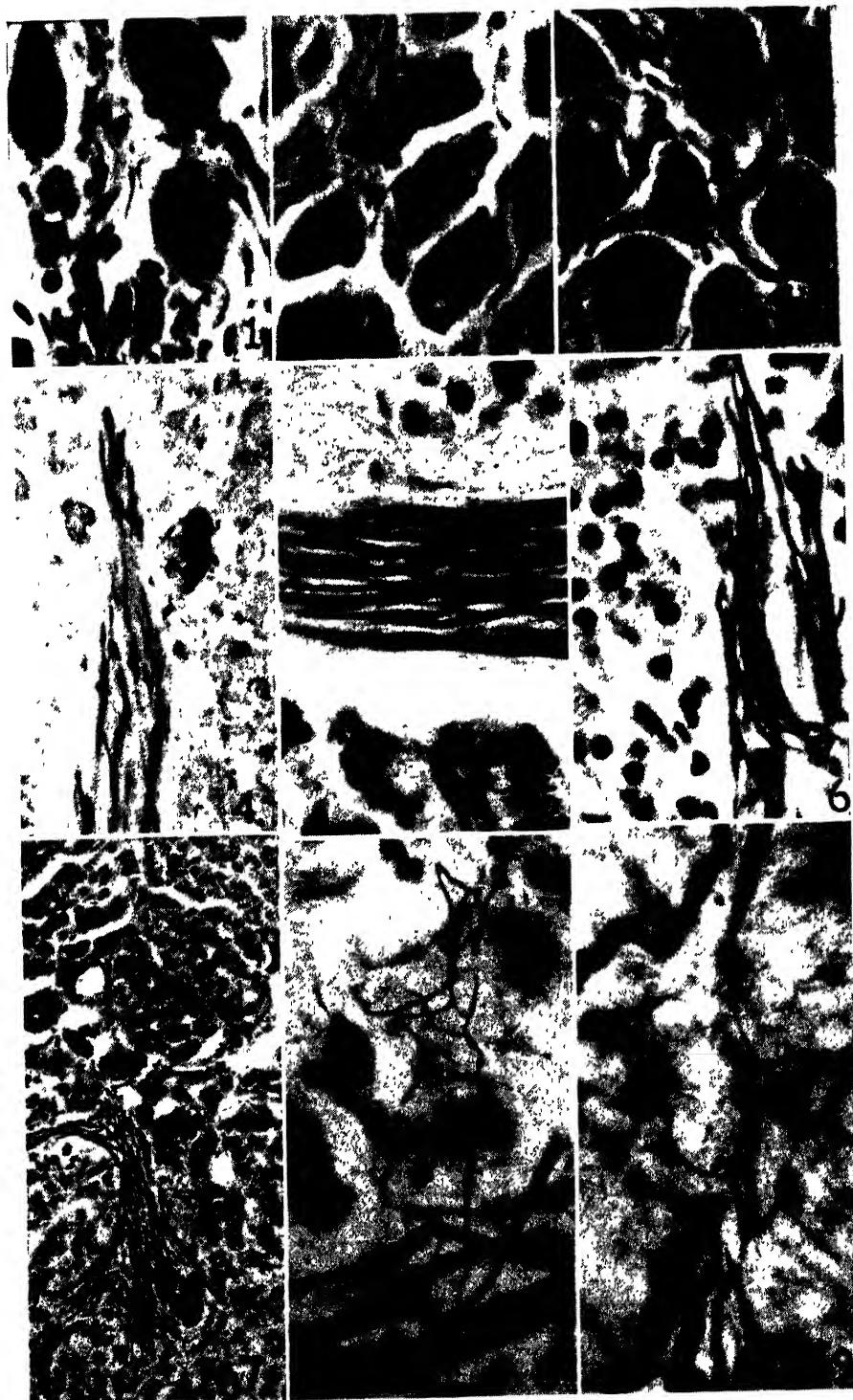
Fig. 6. Intracortical fibers in dog; protargol, fumaric acid, hydroquinone.

Figs. 4, 5 and 6, $\times 500$.

Fig. 7. Fascicle at the cortico-medullary junction in cat; thallium nitrate, protargol, oxalic acid, hydroquinone. $\times 145$.

Fig. 8. The same slide as Fig. 7 except photographed at a magnification of 1000 to show the just resolvable pericellular fibers in the medulla.

Fig. 9. Similar to Fig. 8, excepting that the pretreatment was with lead nitrate instead of thallium and the specimen is the adrenal medulla of dog. $\times 1000$.



silver staining technic which has been worked out for a different type. The arbitrary procedure given in the abstract of this paper may succeed with glandular tissue other than the adrenal gland, but we have not tried it with other glands. The sympathetic ganglia adjacent to the gland stained well when the medullary fibers stained well (Figs. 2 to 5), hence it should be suited to sympathetic nerves elsewhere.

Acid differentiation in a dilute (0.1%) aqueous solution of an organic acid between the steps of protargol impregnation and reduction contributed much toward successful staining of fine nerve fibers in the adrenal medulla. Without the acid treatment, the tissue reduced to a dense brown, but with increasing degrees of acid differentiation background colors ranging progressively thru reddish brown, pink, lavender and blue were obtained. The color served as a guide to the time required in the acid bath. Reddish pink medullary tissue with lavender cortex and black nerve fibers was optimum.

The question of the value of mordants preceding protargol impregnation is not settled but rather merely raised by these experiments. In view of the results obtained with lead or thallium nitrate (Figs. 7 to 9), they seemed to have improved the impregnation of fine nerve fibers and warrant the recommendation of their use. There may be other salts which would influence the selectivity of protargol.

When fixation was made with formamide-chloral-hydrate solution, the use of copper with the protargol bath was optional, and when used, was preferably added later. On the basis of some earlier experiments it appeared that the addition of copper was essential to nerve fiber impregnation after fixation in formalin.

The modifications which differed from earlier protargol methods consisted of mordanting with lead or thallous nitrate solution in a paraffin oven preceding protargol impregnation, and the use of a carefully controlled acid differentiating bath after the impregnation.

Note: Sections should be affixed to the slides as flat as possible and be dried thoroly to minimize loosening during the rather long staining process.

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AN IMPROVED TRIMMER FOR PARAFFIN BLOCKS

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In January 1937, the author published in *Stain Technology* a description of a simple trimmer for paraffin blocks. Since that time so many requests have been received for dimensions and diagrams that it was decided to build a new instrument incorporating several additional features and eliminating certain characteristics which had proved to be undesirable.

The object of the instrument (Fig. 1) is to trim the paraffin block so that the opposite edges will be strictly parallel, thereby preventing the ribbon from curling as it comes off the microtome. This is of particular advantage when using small blocks or when serial sections are desired. While the original design was satisfactory in this respect, there were some undesirable defects. The most outstanding of these was the absence of any device to compensate for side motion when the sliding parts should become worn.

The new instrument has a T-shaped bed made of cast iron. The moving parts are of bronze and are mounted on the upper surface of the bed within steel key-ways, and fastened by machine screws. One side of each key-way is fastened solidly to the bed by flat-head bright, machine screws. The opposite side is attached by cylindrical head screws which pass thru oversize holes permitting compensatory adjustments when side motion, occasioned by use, appears. The blades are made from suitable lengths of stainless steel strips which are ground and honed to a razor edge. These blades are mounted upon the blade carriers by means of set screws. The holes in the blades are also oversize to permit adjustments. The blade carriers are moved into position by means of finely threaded screws which have large knurled heads; the carriers are tapped and threaded to receive these screws.

The microtome pedestal is mounted in a sleeve which permits the paraffin block to be rotated thru 90 degrees. This insures squaring of the block so that the sections will spread evenly and without cracking when placed upon a warm stage or floated upon warm water. The sleeve in turn is mounted in a carrier which is attached to a brass plate by a single large shoulder screw. This permits the carrier to be oriented with reference to the blades. The whole assembly runs in the keyway perpendicular to the blades.

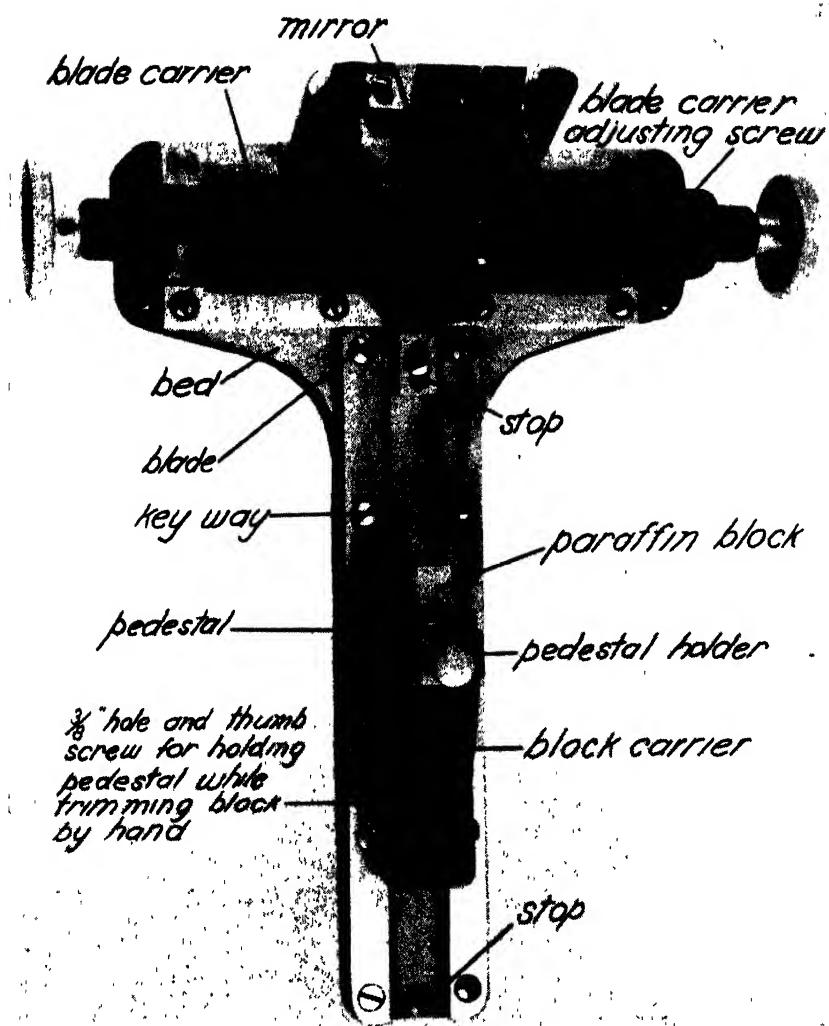


Fig. 1. Photograph of instrument with a paraffin block in position for trimming.

It is advisable to trim the block by hand before using the trimmer. To facilitate this a hole with an accompanying thumb screw is drilled vertically on the back of the carrier. The pedestal can thus be held

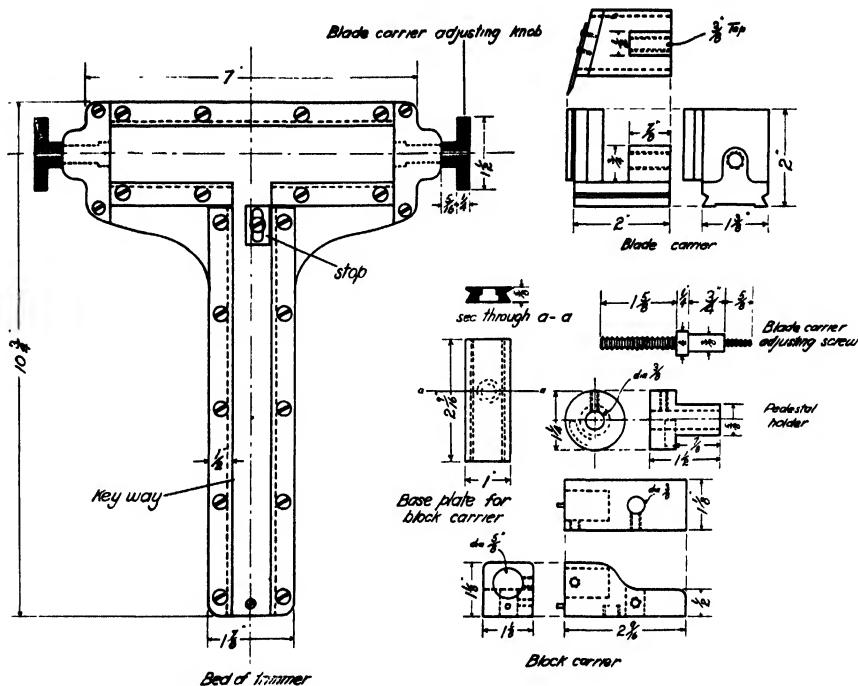


Fig. 2. Working drawing showing all important dimensions.

solidly and in a vertical position while the block is being trimmed by hand.

A stainless steel mirror is mounted on the front of the instrument. This permits the operator to observe the position of the tissue in the block.

The author wishes to express his appreciation to Mr. Carl Sprigle, instrument maker of the Georgetown University Department of Physics, for aid in construction.

STAINING SCAB ACTINOMYCES IN POTATO TUBER TISSUES

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ABSTRACT.—*Actinomyces* hyphae imbedded in the middle lamellae of potato tuber cells may be stained in sections by the use of a modified Gram's stain. The modifications are: a very strong (5%) solution of crystal violet in anilin oil; a 24-hour exposure to both the dye and the iodine solution; and a slow decolorization in absolute alcohol until no more color flows.

Potato scab *Actinomyces* filaments may be readily traced in pieces of the thin skin stripped from small tubers, mounted whole in 10% glycerin without cover glass, allowed to dry for several days with 10% glycerin, with 50% glycerin finally added. The mycelium secretes its typical dark-brown pigment into the adjacent cell walls and can be followed.

The location of the filaments in tuber sections presents such difficulties that no complete account has yet been given of the extent to which they penetrate the tubers, nor their location in the cells.

Potato-infesting *Actinomyces* species are Gram-positive in the living portions of the mycelium; the dead regions are Gram-negative.

Stains, such as Flemming's triple, are not differential, in that they stain nucleus, cytoplasm, and cell walls, as well as any enclosed parasites. The desired stain will affect the parasite alone so that the plant tissues are left unstained. Gram's stain offered possibilities since *Actinomyces* filaments from cultures retain the stain even after a long wash in 95% alcohol. Tests made on microtome sections of young potato tubers following the ordinary Gram method resulted in complete decolorization both of potato tissue and of any parasitic filaments, if the stain solution was that ordinarily used and if the time was the same or longer.

A trial was then made of Jones' method for Gram-positive bacteria.¹ This method is really the triple stain plus the application of Gram's iodine solution and a very strong gentian violet solution. Results with us were still unfavorable, the parasite seemed to decolorize as readily as the host tissues. The use of the strong gentian violet solution of Jones' stain was retained but with the following procedure.

¹Jones, F. R. Winter injury of alfalfa. *J. Agr. Research*, 37, 189-211. 1928.

Small potatoes preserved in 50% alcohol (or Allen-Bouin's mixture) were imbedded in paraffin and sectioned about 8-10 μ thick. The mounted paraffin sections were handled as follows:

1. Treatment with xylol, absolute alcohol, 95% alcohol, and then washing in water.
2. Staining in the gentian violet (crystal violet) solution for 24 hours. This solution was made as follows: 10 cc. of 95% alcohol and 2 cc. of anilin oil were shaken thoroly and then 88 cc. of distilled water added. In this mixture, 5 g. of crystal violet (as small crystals as possible) were dissolved. The solution was shaken thoroly, allowed to stand for a day and then filtered.



Fig. 1. Section of young potato tuber cork layer; *Actinomyces* filaments stained blue, host walls, colorless. $\times 500$.

3. Washing in water to remove excess stain.
4. Immersion in Gram's iodine solution (iodine 1 g., KI 2 g., distilled water 300 cc.) for 24 hours.
5. Washing in absolute alcohol until no more color flowed out of the sections.
6. Immersion in xylol until no further color was drawn from the sections. If none appeared, the sections were mounted in Canada balsam dissolved in xylol.

This preparation of the solutions follows Rawlins' directions² rather than those of Jones, who simply states, "the sections were stained according to Gram's method as described in manuals of bacteriology except that each slide was dipped in water after immersion in the iodine solution to prevent the appearance of iodine crystals after dehydration."

Weigert's Gram stain,³ used for differentiation of Gram-positive bacteria in animal tissues, one part of anilin oil to two parts of xylol, was tried; but the action was so rapid that all gentian violet was removed. It could not be poured on and off the sections without complete destaining. Decolorization in absolute alcohol required up to an hour which gave an opportunity to control its action.

The results were satisfactory in the large majority of trials. Two difficulties were encountered: (1) A complete bleaching of the sections, altho the cells were infected; cause still undiscovered. (2) Gentian violet continued to run in small amounts into the balsam, altho decolorization had been apparently complete in absolute alcohol, making the balsam surrounding the sections a faint purple. The organisms could, however, be seen clearly differentiated in the tuber cell walls.

In the best preparations the organisms were a deep, bluish-black with the host cell walls unstained. The walls of the cork layer had a faint yellow tinge and could be readily seen without a counter-stain, so none was used.

The hyphae of the organism push their way thru the middle lamellae of the potato cells, as has been noted by other investigators and as can be readily seen in views of surface mounts. In stained sections, this relation of potato cell walls to the hyphae is not so clear; stained portions seem to be cell walls but are really the enclosed hyphae; while unstained portions occur where the hyphae have twisted out of focus or the branches of the hyphae inside the walls have been cut off in sectioning.

The hyphae vary considerably in diameter and some are so fine as to be almost on the line of visibility with the oil immersion lens. These very fine filaments decolorize if the destaining is carried too far while the larger ones retain their color. The composition of the enclosing potato cell walls has also an effect on the rapidity of the de-staining process. Cork walls are relatively impermeable and the

²Rawlins, T. E. *Phytopathological and Botanical Research Methods*. Wiley, New York. 1933.

³Weigert, C. Über eine neue Methode zur Färbung von Fibrin und von Mikroorganismen. *Fortzchr. d. Med.*, 5, 228-32. 1887.

rather large hyphae enclosed in them make destaining rather slow. The cellulose walls of the body of the tuber do not protect the delicate hyphae enclosed and destaining is more rapid. The consequence is that a single preparation is very likely to be better either for the large hyphae imbedded in the cork cell walls or for these deeper in the tuber, altho preparations may be obtained where all *Actinomyces* hyphae retain a brilliant color.

EFFECT ON TISSUE VOLUME OF VARIOUS METHODS OF FIXATION, DEHYDRATION, AND EMBEDDING.¹

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ABSTRACT.—A new indirect method is described for following volume changes of homogeneous pieces of tissue during fixation, dehydration and embedding, and area changes during sectioning, staining and mounting. Pieces of rabbit kidney cortex were compared after fixation in Destin's, Orth's, Petrunkevitch's cupric-paranitrophenol, Bouin's, SUSA, Zenker-formol, 10% formalin in distilled water, formalin in saline, Burke's pyridine formalin, CaCO_3 -neutralized formalin, MgCO_3 -neutralized formalin, Bensley's vacuum distilled neutral formalin in distilled water, and Bensley's neutral formalin in saline; during dehydration in ethyl alcohol, dioxan, and tertiary butyl alcohol and clearing in xylol and chloroform; and after infiltration and embedding with parowax, with paraffin-nitrocellulose double embedding technic, with hot nitrocellulose, and with cold nitrocellulose. The H-ion concentration of these fixatives was followed during tissue fixation.

Altho all fixatives showed specific differences, SUSA and Bouin's gave the best general results and neutral formalin mixtures, especially pyridine-formalin, the poorest. Isotonic saline was found superior to distilled water as a formalin diluent, reducing tissue swelling during formalin fixation. Reagents producing marked decreases in tissue volume render such tissues less susceptible to shrinkage during subsequent procedures. Shrinkage of tissue during dehydration and infiltration with hot paraffin may exceed that produced by fixation alone. Excessive heat causes tissue distortion and shrinkage. Infiltration with hot paraffin causes considerable shrinkage, with hot nitrocellulose less, and with cold nitrocellulose the least shrinkage.

In the preparation of microscopical sections living tissues undergo certain changes. These vary with different methods employed. The following experiments were undertaken to compare some of the main factors concerned in these changes.

¹Appreciation is expressed for the suggestions of Professors H. Kirkman and J. E. Markee of the Department of Anatomy and Mr. J. G. Beckerley of the Department of Physics at Stanford University.

²At present at the Barnard Free Skin and Cancer Hospital, St. Louis, Missouri.

METHODS OF VOLUME DETERMINATION

For the studies on changes in tissue volume, the cortex of rabbit kidney from which the capsule had been stripped was cut into pieces about $0.5 \times 0.5 \times 0.3$ cm. By using small pieces of this tissue, complete, uniform, and rapid penetration of fluids was obtained. The change in volume of the tissue was determined indirectly by observing the change in area of one surface of the block. With an unaltered optical system (Fig. 1) consisting of a low power compound microscope, giving an initial magnification of ten diameters, and a camera lucida, successive tracings were made of the same surface of the tissue block

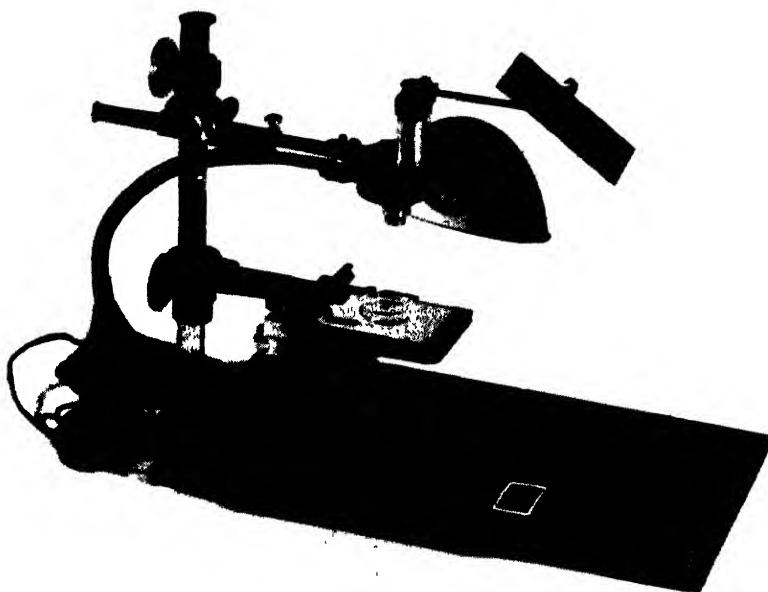


Fig. 1. Apparatus for indirect volumetric comparisons.

barely covered by different fluids. To minimize and standardize the spherical aberration, the block of tissue was centered and oriented in the optical field in the same position each time with the assistance of an eyepiece micrometer and a mechanical stage which held a glass plate upon which rested the dish of fluid with the immersed tissue. To improve the definition of the camera lucida image, the drawings were made with white pencil upon black paper. The area of the tracings was found with the aid of a planimeter, and a volume factor for determining the volume change for any given piece of tissue was obtained by taking the area reading of the planimeter to the $3/2$

power. It is desirable to convert the area readings to a volume factor because the changes in area are not the same as the changes in the volume.

Since for valid results, changes in the tissue block dimensions must be uniform, care was taken to use only blocks which did not change in shape. The constancy of the optical system was checked frequently by making tracings of a standard object. Changes in the thickness of the tissue blocks resulted in changes in magnification of the optical system due to the variation in distance from the objective lens to the tissue block. Consequently, at the conclusion of the experiment the thickness of the firm tissue blocks was determined with fine calipers, and previous area readings corrected accordingly. The thickness of the tissue at previous area readings was found by using a nomograph solving the equation, $\log A_1/\log A_2 = \log T_1^2/\log T_2^2$, in which A refers to area and T to thickness of the tissue, with the subscript numbers designating the various area readings. T_1-T_2 gave the change in tissue thickness. The variation in magnification per millimeter change in thickness was found by experimentally altering the distance from the objective to the object. Usually three area tracings were made of each of two to five comparable tissue blocks at each stage of the experiment and the average of two or three planimeter determinations of each of the six to fifteen tracings taken as the area.

The indirect method of determining volume change is especially adaptable to use upon small pieces of homogeneous tissue for which accurate direct volume determining apparatus is not available. Tarkhan (1931) and others have mentioned the fact that if tissues are permitted to dry between fluid changes, they show a greater shrinkage. With this method the tissues are always covered by the proper fluid, and volume comparisons can even be made in melted paraffin, celloidin, balsam, or any other clear or semi-clear liquid or solid medium. By keeping the covering media at a constant, minimal thickness, it is not necessary to make a correction for differences in refractile properties of the media. The coefficient of variability was 2.80 for the volume changes of twelve pieces of tissue (Fig. 3; No. 1, 2, 6, and 7) during formalin fixation.

Comparing the newly presented indirect method of volume determination with the direct method using displacement by tissues of fluid in a burette, four pieces of rabbit kidney cortex were found to have volumes, as compared with their fresh volumes, of $140 \pm 3\%$ after fixation in 10% formalin, of $102 \pm 12\%$ in 70% alcohol, and of $60 \pm 6\%$ after embedding in parowax. Volume values were taken

TABLE I. THE pH READINGS, TO THE NEAREST 0.05 pH, OF VARIOUS FIXING FLUIDS, EACH CONTAINING ONE-TWENTIETH OF ITS WEIGHT IN DOG KIDNEY

| Fixing Fluid | Fresh sol. pH | pH-value at various periods after fixation started | | | | | | | | | |
|------------------------------------------------------|---------------------|----------------------------------------------------|------|------|------|------|------|------|------|------|------|
| | | Hours | | | | | Days | | | | |
| 1 | 2½ | 5 | 8 | 18 | 26 | 49 | 6 | 17 | 95 | 175 | |
| 10% formalin (Baker's) in distilled water | 4.35 | 4.70 | 5.20 | 5.40 | 5.45 | 5.45 | 5.60 | 5.60 | 5.10 | 4.85 | 4.40 |
| 10% formalin in 0.9% sodium chloride | 4.35 | 4.80 | 5.10 | 5.15 | 5.15 | 5.15 | 5.10 | 4.80 | 4.60 | 4.40 | |
| Burke's pyridine formalin (25%) | 8.20 | 7.90 | 7.60 | 7.80 | 7.75 | 7.70 | 7.70 | 7.60 | 7.55 | 7.25 | |
| CaCO ₃ 10% neutralized formalin | 6.60 | 6.30 | 5.90 | 5.95 | 5.90 | 5.85 | 5.80 | 5.75 | 5.40 | 5.05 | 4.85 |
| MgCo ₃ 10% neutralized formalin | 7.15 | 6.70 | 6.20 | 6.20 | 6.10 | 6.05 | 6.00 | 5.95 | 5.60 | 5.20 | 4.95 |
| 10% Bensley's neutral formalin | 6.85 | 6.35 | 6.40 | 6.25 | 6.25 | 6.15 | 6.15 | 6.10 | 5.70 | 5.25 | 5.00 |
| 10% Bensley's neutral formalin in saline | 6.90 | 6.25 | 5.80 | 5.80 | 5.75 | 5.75 | 5.60 | 5.40 | 5.10 | 4.85 | 4.70 |
| 10% formalin (Merck's) in distilled water | 6.50 | 6.45 | 6.00 | 6.00 | 5.90 | 5.90 | 5.80 | 5.80 | 5.30 | 5.15 | 4.60 |
| Destin's fluid | 3.90 | — | — | 3.80 | 3.80 | 3.80 | 3.85 | 3.85 | 3.90 | 3.90 | 3.85 |
| Orth's fluid | 4.00 | — | — | 4.20 | 4.50 | 4.50 | 4.80 | — | 5.00 | — | — |
| Zenker-formol | 3.70 | 3.85 | 4.00 | 4.00 | 4.10 | 4.20 | 4.15 | 4.15 | 4.25 | 4.25 | — |
| Bouin's fixative | 2.00 | — | 2.00 | 2.00 | 2.00 | 2.05 | 2.00 | 2.05 | 2.05 | 2.05 | 2.05 |
| SUSA fixative | 1.90 | 1.90 | 1.90 | 1.90 | 1.90 | 1.90 | 1.15 | 1.15 | 1.20 | 1.20 | — |

from the average of two separate burette readings, which checked within 0.02 cc., the volume of the fresh block varying from 0.14 cc. to 0.30 cc. A repetition of the determination for the average volume of five pieces of fixed tissue checked within 1%.

The kidney cortex after removal from the rabbit was kept in fresh rabbit serum for the short time before the camera lucida tracings of the fresh tissue blocks could be completed. The experimental work on volume was conducted in two separate parts, the second being performed several months after the first, using tissues from a different animal and different reagents; hence the two parts are not directly comparable. All tissue samples for each part of the experiment were taken from the same animal to insure uniform consistency.

2. pH CHANGES OF FIXING FLUIDS DURING TISSUE FIXATION

Several freshly excised pieces of dog kidney totaling about 2.5 cc. were weighed to within 0.02 g. and placed in twenty times their volume of fixative in chemically clean glass-stoppered vials. The pH-values of fixing fluids (Table 1) were taken with a glass electrode just before the tissues were dropped into them, at intervals during fixation, and, in some cases, for 6 months subsequently.

During formalin fixation the changes in pH were greatest within the first 5 hours. During the first 48 hours the pH-value of all solutions tended to approach a common median point, probably the reaction of the tissue, altho the solution containing pyridine had the greatest buffer capacity and was altered least. After several days, however, the pH-value of all solutions started to fall slightly, probably from the breakdown of the formalin to give acidic compounds such as formic acid.

Zenker-formol and Orth's fixatives showed a moderate increase in pH. SUSA, Bouin, and Destin³ showed relatively little change in their low pH-values.

3. VOLUME CHANGES DURING VARIOUS FIXATIONS AND DEHYDRATIONS

In the first part of the experiment on changes in tissue volume, the results of which are shown in Table 3 and Fig. 2, several samples were fixed in each of the following fluids: Petrunkevitch's No. 2 cupric-paranitrophenol, Bouin's, SUSA, Destin's, Orth's, Zenker-formol, and 10% formalin, dehydrated thru the alcohols to xylol, and embedded in parowax. Formalin-fixed groups were also dehydrated and cleared by each of the tertiary butyl alcohol, chloroform, and

³Dustin (?). Formula used in this study was taken from McClung, "Microscopical Technique," second edition.

dioxan methods. To get maximal effects of the various agents, the tissues were left for what was considered to be an ample length of time in each solution.

The tissue blocks were treated separately according to the following schedules. Tissues, after 16 hours in Petrunkevitch's fixative, were washed in 70% alcohol. After 18 hours in Bouin's, the tissue was transferred directly to 70% alcohol and given several changes. After 6 hours in Heidenhain's "SUSA" fluid, the tissue was washed in 70% alcohol. Tissue was fixed 7 days in Destin's fluid, washed 2 hours in distilled water, and upgraded to 70% alcohol. Following 5 days fixation in Orth's fluid, the tissue was washed 24 hours in running water and dehydrated to 70% alcohol. Samples were fixed 6 hours in Zenker-formol, washed in running water 16 hours, and upgraded to 70% alcohol. Following 48 hours fixation in 10% Merck's "neutral" formalin in distilled water, the tissues were washed 3 hours in running water and dehydrated to 70% alcohol. All tissues were left 24 hours or longer in 70% alcohol before further dehydration.

TABLE 2.— TERTIARY BUTYL ALCOHOL DILUTION TABLE (AFTER JOHANSEN)

| | Percentages of TBEA | | | |
|----------------------------------|---------------------|--------|--------|--------|
| | 70% | 85% | 95% | 100% |
| Distilled water | 80 cc. | 15 cc. | — | — |
| 95% ethyl alcohol | 50 cc. | 50 cc. | 45 cc. | — |
| Tertiary butyl alcohol | 20 cc. | 35 cc. | 55 cc. | 75 cc. |
| Absolute ethyl alcohol | — | — | — | 25 cc. |

The samples which were to go thru xylol were left 10 hours in 85% alcohol, 12 hours in 95% alcohol, a total of 4 hours in three changes of absolute alcohol, 1 hour in equal parts of absolute alcohol and xylol, a total of 3 hours in three changes of xylol, and 12 hours in xylol saturated with paraffin at 38°C. Tissues infiltrated with paraffin by the chloroform method were treated in the same way except that chloroform was substituted for xylol in the above mixtures. Samples were transferred from 70% alcohol to three changes of dioxan totaling 10 hours and then into a mixture of equal parts of dioxan and parowax for 12 hours at 50°C.

The tertiary butyl alcohol (TBA) schedule (table 2) provided for 24 hours in 70% tertiary butyl and ethyl alcohol (TBEA) mixture following transfer from 70% ethyl alcohol, 2 hours in 85% TBEA mixture, 2 hours in 95% TBEA, 2 hours in 100% TBEA, a total of 4 hours in three changes of pure TBA, 1 hour in a mixture of equal parts of TBA and paraffin oil, and then infiltration with parowax. This was done in the method suggested by Johansen (1940) by immersing the tissues in sufficient TBA-paraffin oil mixture on top of melted parowax, three-fourths filling shell vials which had been cooled enough to solidify the parowax. The vials were placed in the oven at 50°C. for 12 hours, and the tissues slowly settled as the parowax melted, thereby giving a gradual infiltration with parowax. The tissues were subjected to slightly greater heat than usual so as to show the shrinking effect of the heated reagents. All tissues during infiltration with parowax were given four changes of pure parowax totaling 5 hours at 52°C. and then embedded.

The TBA had a flash point of 70°, melting point of 17.5°C., and was obtained from California Botanical Materials Co., 787 Melville Ave., Palo Alto, Calif. The dioxan was from General Biological Supply House, Chicago, Ill.

4. VOLUME CHANGES WITH VARIOUS FORMALIN FIXATIONS AND EMBEDDINGS

In the second part of the experiment on changes in tissue volume, the results of which are shown in Table 4 and Fig. 2, three samples were fixed in each of the seven following fluids: 10% formalin in distilled water, 10% formalin in 0.9% aqueous NaCl, as suggested by Mann (1902, p. 92) and Carleton (1922), Burke's (1933) pyridine neutralized 25% formalin, CaCO₃-neutralized 10% formalin, MgCO₃-neutralized 10% formalin, Bensley's (1938, p. 43) neutral 10% formalin, and Bensley's neutral 10% formalin in 0.9% sodium chloride. Samples of formalin fixed tissue were embedded by each of the parowax, double embedding, hot nitrocellulose, and cold nitrocellulose technics.

The nitrocellulose used was made by Hercules Powder Company, Parlin, New Jersey and was labeled R. S. $\frac{1}{2}$ second, viscosity 3/20. Altho "neutral" reagent formaldehyde of Merck & Co., Rahway, New Jersey, was used in the first part of the experiment, in the second part the C.P. analyzed formaldehyde of J. T. Baker Chemical Co., Phillipsburgh, New Jersey, was used as a stock solution. The same schedule for formalin fixation, alcohol dehydration, xylol clearing, and parowax infiltration and embedding was followed in all experiments. In the double embedding schedule tissues were dehydrated into absolute alcohol by the standard method and left in equal parts absolute alcohol and ether for 18 hours. After being transferred to 5% nitrocellulose for 3 days, they were put into a mixture of equal parts of 10% nitrocellulose and pure clove oil for 24 hours. The tissues were then dropped into two changes of chloroform of one-half hour each, into benzol for one hour, and then into benzol saturated with parowax at 38°C. for 15 hours. Infiltration was completed by four changes of parowax totaling 5 hours at 52°C.

In the hot nitrocellulose technic tissues were run thru absolute alcohol and ether as for double embedding and then thru three grades of nitrocellulose at 56°C. according to the specifications of Koneff and Lyons (1937) except that the times in the solutions were doubled because of the size of the tissues and the desire to permit ample time for determining the effects of the hot nitrocellulose.

With the cold nitrocellulose schedule, the tissues were upgraded thru absolute alcohol and ether as for double embedding and then thru solutions of 5, 10, and 20% nitrocellulose with 3 days in each. Tissues were embedded in 20% nitrocellulose, exposed to chloroform vapor for 2 hours, immersed in chloroform for 4 hours, and stored in 80% alcohol.

Using a modification of the apparatus shown in Fig. 1, in which the objective stage was moved to maintain the object surface at a constant distance from the objective lens despite the varying depth of the object, the area of the cut surface of one nitrocellulose block (Fig. 2, No. 2) was compared with that of the last three 16 μ sections cut from it. Under standard optical conditions, as compared with the area of the block surface covered with 80% alcohol and a cover slip, the cut sections showed an area of 92±0% in 80% alcohol, of 82±0%

TABLE 3.—AVERAGE VOLUME PERCENT, AS COMPARED WITH FRESH TISSUE, OF RABBIT KIDNEY DURING FIXATION, WASHING, DEHYDRATION AND CLEARING, AND PAROWAX INFILTRATION AND EMBEDDING

(The number of samples in each group is shown in parentheses. The variation is expressed as the difference between the average volume percent and the greatest deviation from the average)

| Procedure (see p. 72) | Fixed tissue | Washed tissue | Tissue in 70% alc. | Cleared tissue | Embedded tissue |
|-----------------------------------|-----------------|------------------|-----------------------|-------------------|--------------------|
| Petrunkewitch-xylol | 117±2(3) | _____ | 130±1(3) | 79±1(3) | 64±4(3) |
| Bouin-xylol | 86±1(2) | _____ | 82±0(2) | 71±3(2) | 63±2(2) |
| SUSA-xylol | 82±2(4) | _____ | 83±4(4) | 70±3(4) | 58±2(4) |
| Destin-xylol* | 104±3(3) | 102±2(3) | 91±3(3) | 71±2(3) | 56±4(3) |
| Orth-xylol | 71±3(3) | 67±3(3) | 69±2(3) | 64±1(3) | 55±3(3) |
| Zenker-formol-xylol | 70±4(4) | 68±4(4) | 61±5(4) | 54±4(4) | 47±1(4) |
| 10% formalin TBA | 129±2(3) | 125±3(2) | 78±2(3) | 68±2(3) | 60±3(3) |
| 10% formalin-chloroform | 133±2(4) | _____ | 85±4(4) | 74±3(4) | 58±5(4) |
| 10% formalin-xylol† | 134±4(4) | 126±1(2) | 82±4(5) | 64±2(4) | 56±3(5) |
| 10% formalin-linylan | 140±5(4) | _____ | 84±5(4) | 74±5(4) | 60±1(4) |

*Destin-xylol: in xylol-parowax, 67±1(2); in parowax 1½ hr. at 52° C., 63±1(2); in parowax 5 hr. at 52° C., 60±1(2).

†10% formalin-xylol: in absolute alcohol, 74±4(5); in parowax 5 hr. at 52° C., 57±3(3).

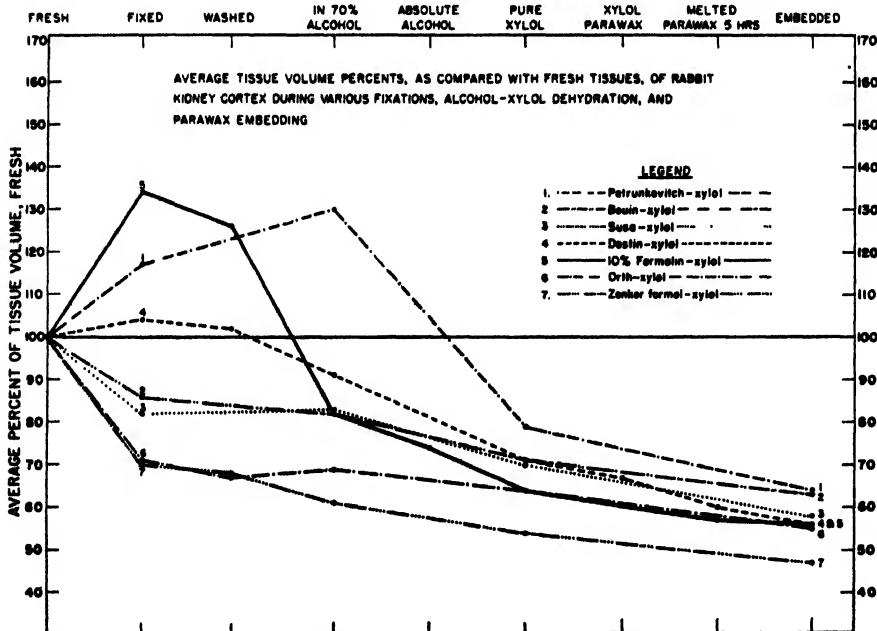


FIGURE 2

in xylol after staining the sections without fastening to slides, and of $82 \pm 0\%$ after being mounted 16 hours in neutral balsam at 40°C .

A similar comparison between a block of formalin-fixed parowax-embedded kidney and five 8μ sections cut from it did not show any significant change in area. By the same method a decrease in area of $28 \pm 0\%$ was found in comparing three 3μ mounted stained sections of Destin-fixed, TBA-dehydrated rat kidney with their block embedded in 62° paraffin. The tracings showed twice as much decrease in width as in length suggesting that compression during sectioning was an important factor, altho it was not evident macroscopically or microscopically.

Sections, 8μ thick, were cut at $25-28^\circ\text{C}$. from all groups of three comparable blocks in the first part of the experiment and in the second part from single, promptly fixed pieces submitted to each separate schedule, eliminating any difference in the microscopic picture due to varying delay before fixation. Altho several months elapsed between the time the tissues in the first part of the experiment were embedded and the time they were sectioned, all other blocks were sectioned a few days after embedding. Care was taken thruout the work to seal all blocks not being used by dipping in liquid parowax.

The relative ease with which about fifteen sections could be cut at a uniform speed from blocks previously trimmed and oriented for ready sectioning, was studied, especial attention being paid to the sound produced by the microtome knife passing thru the tissue. Since these small, well infiltrated tissues cut without difficulty, a somewhat dull knife was used in an attempt to detect maximal differences in sectioning and in tissue hardness. Since Aumonier (1938), in studying the distortion of tissues by sectioning, found the sharpness of the knife to be the chief variable, numerous blocks were sampled at the same place on the knife so that the sharpness of the knife would be relatively uniform.

To eliminate personal bias in judging ease of sectioning or the microscopic picture, blocks were assigned numbers and their identity obscured until the final results were tabulated. This comparison between blocks was repeated, varying the order of sectioning, so that each block was tested three times and rated according to ease of sectioning. Results for each method of treatment were averaged and arbitrarily divided into fairly distinct groups (Table 5).

Sections from each of the paraffin blocks were mounted and stained simultaneously in the same staining dish with Harris' hematoxylin and eosin and with Heidenhain's azocarmine modification of Mallory's triple connective tissue stain (Mallory, 1938). Sections were cut

TABLE 4.—AVERAGE VOLUME PERCENT, AS COMPARED WITH FRESH TISSUE, OF RABBIT RENAL CORTEX DURING FORMALIN FIXATION, ALCOHOL-XYLOL DEHYDRATION, AND EMBEDDING

(The number of samples in each group is shown in parentheses. The variation is expressed as the difference between the average volume percent and the greatest deviation from the average)

| Procedure (see p. 73) | Fixed tissue | Tissue in 70% alc. | Absolute alcohol | Tissue in benzol | Embedded tissue |
|----------------------------------------------------------|-----------------|-----------------------|---------------------|---------------------|--------------------|
| Formalin-cold-nitrocellulose | 140±4(3) | 108±2(3) | 98±1(3) | — | 92±2(3) |
| Formalin-hot-nitrocellulose | 140±2(3) | 105±2(3) | 94±1(3) | — | 79±4(3) |
| Bensley's neutral formalin-parowax | 166±7(3) | 119±5(3) | — | — | 74±2(2) |
| CaCO ₃ neutralized formalin-parowax | 148±2(3) | 108±2(3) | — | — | 72±2(3) |
| MgCO ₃ neutralized formalin-parowax | 146±5(3) | 105±2(3) | — | — | 68±3(3) |
| Formalin-parowax | 143±2(3) | 109±3(3) | — | — | 68±4(3) |
| Formalin double-embedding | 141±5(3) | 104±3(3) | 97±2(3) | 88±3(3) | 68±4(3) |
| Bensley's neutral formalin in saline-parowax | 130±6(3) | 93±4(3) | — | — | 64±2(3) |
| Formalin in saline-parowax | 119±0(3) | 87±4(3) | — | — | 55±2(3) |
| Burke's pyridine-formalin-parowax | 170±0(2) | 75±2(3) | — | — | 48±2(3) |

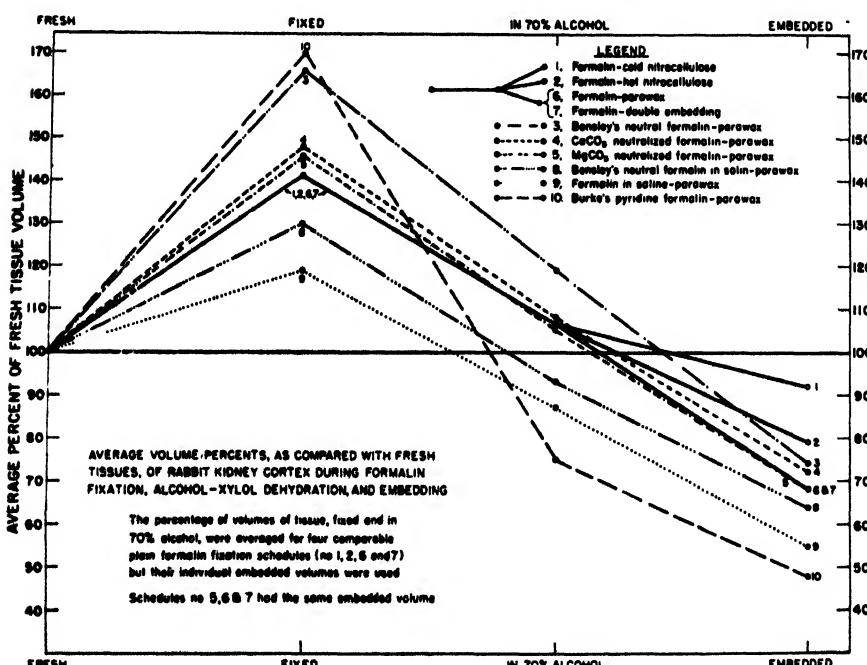


FIGURE 3

from the nitrocellulose blocks at 16, 12, and 8 μ and stained similarly. The sections stained by each method, identified only by number, were carefully studied and graded (Table 5). In many cases tissue sub-

Table 5 — A comparison of the average hardness of tissue blocks and staining properties, fixation and differential shrinkage based (A) on three observations on each of three comparable mounted sections of rabbit kidney fixed and dehydrated by the various methods; and (B) on six observations on one mounted section each of rabbit kidney fixed in various formalin solutions and embedded by different methods.

For each method the hardness of tissue was averaged from three sectionings of each of three comparable blocks in (A) and for three sectionings of one block in (B). H & E is for Hematoxylin and Eosin and MAz for Mallory azan stain. Because of differences in technique sections stained and cut in nitrocellulose are not comparable with parowax embedded material.

| PROCEDURE | MICROSCOPIC PICTURE | | | | | | | | | | TISSUE HARDNESS | |
|-----------|----------------------------------------|-------------|-----------|-------------|-------------|---------------|--------|------------|---------|------|-----------------|--------|
| | NUCLEAR | | | CYTOPLASMIC | | | | SHRINKAGE | | | | |
| | Fixation | H & E Stain | MAz Stain | Fixation | H & E Stain | MAz cytoplasm | MAz ct | Glomerular | Tubular | | | |
| A | Petrunkewitch-xylol | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | Max. | Med | Soft |
| | Bouin-xylol | +++ | +++ | +++ | + | ++ | ++ | +++ | +++ | Min. | Min | Soft |
| | Susa-xylol | +++ | +++ | +++ | ++ | ++ | +++ | +++ | +++ | Min. | Min. | Medium |
| | Destin-xylol | ++ | ++ | +++ | +++ | ++ | ++ | ++ | ++ | Min. | Med. | Medium |
| | Orth-xylol | + | + | ++ | ++ | + | ++ | ++ | ++ | Min. | Max | Hard |
| | Zenker-formol-xylol | +++ | +++ | ++ | ++ | + | ++ | ++ | ++ | Min. | Max | Soft |
| | 10% Formalin-TBA | ++ | +++ | + | + | + | + | + | + | Max | Min. | Medium |
| | 10% formalin-chloroform | ++ | +++ | + | + | + | + | + | + | Max | Min. | Medium |
| | 10% formalin-xylol | ++ | +++ | + | + | + | + | + | + | Max. | Min | Medium |
| | 10% formalin-dioxane | ++ | +++ | + | + | + | + | + | + | Med. | Min | Hard |
| B | Formalin-cold nitrocellulose | +++ | +++ | + | ++ | +++ | ++ | +++ | +++ | Min. | Min | Hard |
| | Formalin-hot nitrocellulose | +++ | +++ | + | ++ | +++ | ++ | ++ | ++ | Min | Min | Medium |
| | Bensley's formalin parowax | +++ | +++ | + | + | +++ | ++ | + | Med. | Med | Medium | |
| | CaCO ₃ neutral form parowax | +++ | +++ | + | + | +++ | ++ | + | Max. | Med | Hard | |
| | MgCO ₃ neutral form parowax | +++ | +++ | + | + | +++ | ++ | + | Max | Med | Soft | |
| | Formalin-parowax | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | Med. | Med. | Medium |
| | Formalin-double embedding | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | Med. | Med | Hard |
| | Bensley's form. in saline-paro | ++ | +++ | ++ | ++ | +++ | +++ | ++ | Max. | Med | Medium | |
| | Formalin in saline-parowax | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | Med | Med | Hard |
| | Burket pyridine form. parowax | + | ++ | + | ++ | ++ | ++ | ++ | ++ | Med | Max | Medium |

jected to the same schedule held the same rank within each group thruout all six or nine total repeated testings. Results were checked by the independent rating of a competent cytologist.

Altho the tissue fixed in Petrunkewitch's fluid stained well, the nuclei appeared swollen and showed less distinct chromatin. This fixation gave the softest blocks for cutting. Bouin and SUSA gave the best all around results, altho their cytoplasmic fixation, especially Bouin's, was not so good. Their picture with Mallory-azan was the most brilliant. SUSA-fixed kidney showed the least differential shrinkage between glomerular loops and Bowman's capsule. By their distinctive glomerular picture in which the hyalin base of the

capillary loops stood out clearly and relatively distended, Destin-fixed sections were readily identified. Kidney fixed in Orth's fluid tended to stain diffusely with anilin blue. Tissues dehydrated with dioxan in all instances stained slightly poorer than formalin-fixed kidney dehydrated by other methods. Longer staining with Mallory-azan of formalin-fixed sections improved the connective tissue staining, but not the nuclear and cytoplasmic differentiation. Kidney fixed in neutralized formalin mixtures showed the poorest Mallory-azan staining in all respects and poorer cytoplasmic pictures than the more acid formalin solutions. The use of normal saline instead of distilled water as a formalin diluent improved cytoplasmic fixation, altho the tissue was somewhat harder for sectioning. Kidney fixed in Bensley's neutral formalin in distilled water showed an especially poor cytoplasmic fixation, but the glomerular picture with clear, open capillaries was definitely superior, resembling Destin-fixed glomeruli. The formalin-double-embedding schedule gave results equal or slightly superior to formalin-parowax in most respects, altho it gave slightly more glomerular shrinkage. Burke's pyridine formalin gave bizarre cytoplasmic swelling, which almost filled the proximal convoluted tubules.

5. GENERAL DISCUSSION

FIXATION

The studies of the fixing agents reported in the literature show considerable variation in results. This is explained in part by the use of different tissues, of various sized blocks, of different schedules, and of reagents of not exactly the same composition.

Hirsch and Jacobs (1926) concluded that various chemical and physical properties of a fixative are more important than its molecular concentration. Altho the present results tend to confirm this finding, formalin solutions in isotonic saline were in general equal or superior to hypotonic formalin fixing solutions. Altho formalin solutions in saline gave less initial tissue swelling during fixation, the reaction of subsequent dehydration and embedding reagents produced shrinkage nearly equal to that of formalin without saline so that the total final shrinkage with saline was greater. This greater tissue shrinkage and density may explain in part the increased hardness on sectioning.

Burke (1931) has discussed the importance of H-ion concentration and the various methods of neutralizing formalin fixatives. Altho, as claimed by Burke, pyridine-formalin has better buffering and shows less change in pH during fixation, it gave outstandingly poor micro-

scopic results upon the kidney. Authorities differ as to the most desirable pH for formalin fixing solutions. Jacquier (1930) working with snail hermaphrodite gland concluded that the best fixation is produced at pH-values much lower than those corresponding to the different isoelectric points of the various cellular elements. Manstein (1934) left mouse tissues, including kidney, in phosphate buffer solutions for 24-48 hours at various temperatures. The karyolytic and staining changes in the nuclei became much more pronounced as the reaction of the buffers was varied from pH 5 to 8. The present results show that, in general, ordinary acid formalin is superior to neutralized formalin for fixation of tissues such as kidney, altho on other tissues and for special purposes this might not be true. Most authors have their own preference as to the most desirable type of neutral formalin and it is very likely that each may have slight advantages. Generally, of the neutral mixtures tried, $MgCO_3$ -neutralized formalin, followed closely by $CaCO_3$ -neutralized formalin, gave the best results.

Sato (1931, I) reported that the higher the pH-value of the tissue the more the swelling. The present results for change in gross volume also show the same thing, with the exception of $CaCO_3$ - and $MgCO_3$ -neutralized formalin.

These studies show that for rabbit renal cortex "formalin shrinkage" is not caused by the fixative, as claimed by some, but by the dehydration and embedding. Several writers have stated that formalin does not seem to fix tissue proteins well, thus permitting alteration by subsequent reagents. For this reason, formalin-fixed tissues were chosen for a comparative dehydration and embedding study. Such tissues show extreme swelling and shrinking and give a poorer, more distorted microscopic picture than do tissues showing less extreme volume changes such as those fixed in SUSA, Bouin's, or Destin's fluids.

Since the renal cortex is made up of cellular and connective tissue elements which react differently to different reagents, as shown by Tarkhan and others, a reagent such as formalin would be expected to cause great differential shrinkage and distortion within the tissue. This was confirmed microscopically, since glomerular shrinkage was most marked with formalin fixed tissues. The fixative which produces the least volume change does not, however, necessarily give the best microscopical picture, for Zenker-formol, which caused the greatest shrinkage, is widely used because of its excellent cytoplasmic preservation. The most distinctive changes in the microscopic pic-

ture and gross volume were due to differences in fixation rather than dehydration. These experiments confirm the statements of others that tissues shrinking greatly during fixation tend to show less change under subsequent shrinking conditions.

DEHYDRATION AND CLEARING

Dioxan, which recently has been hailed by some as the answer to all tissue dehydration difficulties, caused 35% shrinkage during parowax infiltration, which is $3\frac{1}{2}$ times the shrinkage during infiltration after xylol and 5 times the shrinkage during infiltration after TBA. It is possible that less shrinkage might have resulted if the tissue had not been dehydrated to 70% alcohol for purposes of comparison before going into dioxan or if the dioxan used had been dried over CaCl_2 before use, as recommended by some authorities, but not mentioned in some articles on dioxan. The dioxan used was supposedly free from water and satisfactory for tissue dehydration purposes. Johansen (1937) is one of the few writers to condemn, for other than toxicological reasons, the indiscriminate use of dioxan. He mentions that some brands contain at least 10% of water and other impurities which may produce poor paraffin infiltration and shrinkage.

Seki (1937, II) gave experimental evidence for the sluggish penetration and shrinkage effects of dioxan. Upon cow liver, spleen, and tendon, the measured gross shrinkage after dioxan was much greater than after ethyl alcohol. The results given here check with his observation that altho xylol shrinks tissues more than dioxan, in going into hot paraffin the final shrinkage is greater in dioxan treated tissue.

A block of formalin-fixed, dioxan-dehydrated kidney infiltrated in dioxan-parowax at 34°C . shrank less and was twice as soft for sectioning than were two comparable blocks left in the same mixture at 50°C ., altho their microscopic pictures were similar.

The changes from 70% alcohol to xylol produced twice as much shrinkage as the transition from 70% alcohol to TBA, dioxan, or chloroform, all of which produced similar degrees of change. Chloroform permitted more change than xylol while the tissues were being infiltrated with parowax, but this may be explained partially by the greater initial shrinking action of xylol, thus permitting less shrinkage by the hot parowax subsequently. The least shrinkage in passing from 70% alcohol to final embedding and also in passing from complete dehydration to final embedding was obtained with TBA. After experience with all four methods of dehydration employed, it has been found that ethyl-alcohol-chloroform and especially TBA dehydration give the most satisfactory results in general micro-

technical work both in ease of sectioning and in final microscopic quality of tissues. Xylol has been the most unsatisfactory. Other things being equal, it is desirable to get minimal tissue shrinkage during fixation, dehydration and embedding.

EMBEDDING

A comparison of embedding methods shows comparable gross shrinkage with parowax and with parowax-nitrocellulose, less shrinkage with hot nitrocellulose, and slight shrinkage with cold nitrocellulose. Excessive heat, whether in nitrocellulose, dioxan-parowax, or especially in parowax, causes tissue shrinkage and should be avoided.

In cooling from 59° to 20°C., 500 cc. of parowax was found to decrease 14.3% in volume. The paraffin, in shrinking more than the tissue, must exert a compressing force upon it. Since during embedding celloidin itself may be observed to shrink slightly, one would expect the embedded tissue, as observed in the experiments with nitrocellulose, also to shrink slightly.

MICROSCOPIC SECTIONS

Embedded tissue has not necessarily reached its maximum capacity to shrink, so that in sectioning it may be further distorted, and subsequent heat, staining, dehydration, and clearing agents, which caused change in the volume of the tissue block, may result in still further change in the fragile section of tissue. Such changes occur during the sectioning and dehydration of nitrocellulose embedded sections; this has also been shown by Sugita (1917).

Aumonier (1938) found that thinner sections and "wax" of higher melting point gave greater shrinkage of tissues during cutting and fixing sections to the slide. This agrees with the present observation of shrinkage in thin sections embedded in hard paraffin while thicker sections in soft parowax, which were allowed only 30 seconds to straighten over water on a warming plate at 39°C., showed no area change. Sugita (1917, p. 523) also mentioned the importance of temperature while stretching paraffin ribbon and observed that, while at the melting point the section size was unaltered, excessive heat produced much expansion followed by sudden contraction to less than the original size on cooling. Since he was unable to detect an increase in width to compensate for the loss in length, Aumonier concluded that his sections must have increased in thickness. Conversely, altho cut celloidin sections can change in size in all directions, paraffin sections fastened relatively firmly to the slide by albumin would be free to change chiefly in thickness. Since the supporting paraffin for the section of tissue is removed before staining, reagents

should tend to shrink it more than in the case of tissue still supported by celloidin.

Generalized conclusions drawn from the specific volume changes reported in this paper should be made with caution, since these changes may be altered readily by differences in the composition and size of the tissue used, by the procedure followed, and by the reagents employed.

6. CONCLUSIONS

1. The indirect method described in the text has been found satisfactory in following volume changes of homogeneous pieces of tissue during fixation, dehydration and embedding, and area changes during sectioning, staining and mounting.

2. The changes in H-ion concentration of fixing fluids and the great variability of microscopic and volume changes produced upon rabbit kidney by the action of certain fixatives, dehydration and clearing agents, and embedding methods were determined (Tables 1, 3, 4, 5 and Fig. 2, 3).

3. Altho all reagents showed specific differences on rabbit kidney, SUSA and Bouin's fixatives yielded the best general results; and neutral formalin mixtures, especially pyridine-formalin, showed the poorest. The use of isotonic saline diluents slightly improved formalin fixation. Ten percent formalin fixation does not necessarily shrink tissues, but as shown with rabbit kidney cortex, it may cause substantial swelling.

4. Reagents producing marked decreases in tissue volume render such tissues less susceptible to further shrinkage during subsequent procedures. Shrinkage of tissue during dehydration and infiltration with hot paraffin may produce more shrinkage than fixation alone.

5. Excessive heat causes tissue distortion and shrinkage. Infiltration with hot paraffin causes considerable shrinkage, with hot nitrocellulose less, and with cold nitrocellulose the least shrinkage.

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A CONVENIENT COOLING METHOD IN PARAFFIN SECTIONING.

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The cooling of the paraffin block in the preparation of microscope slides is most commonly done with a refrigerator, ice or ice water. During the past year we have utilized CO₂ for this purpose, and have found it superior on the whole to the above methods because it does away with dripping water, requires no refrigerator, and is more rapid. Refrigerators and ice have never offered a really satisfactory solution

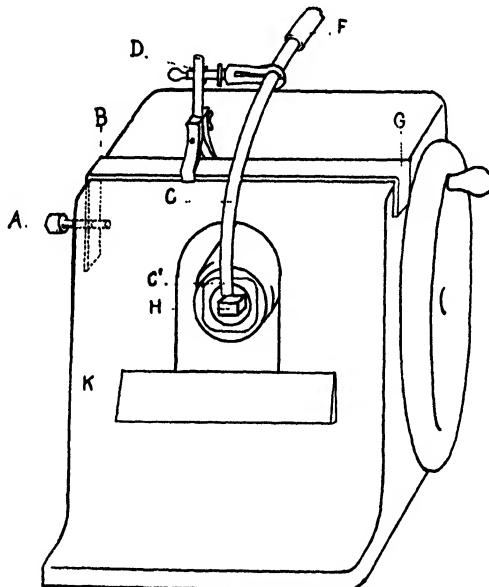


Fig. 1. Sketch of Cooling Apparatus.

(A) Screw which serves to clamp the metal strip (B, G) to the microtome. (C, C') Copper tubing, supported by a burette clamp (D), and connected to a length of rubber tubing (F). (H) Paraffin block. (K) Knife.

to the problem of cutting serial sections. Unless comparatively few sections are to be cut, the block soon becomes warm, and this means the loss of some sections, if the block is removed from the microtome for further cooling. With the device described below, the block may be cooled down as frequently as one desires without disturbing its initial orientation in the microtome. We have found the device so

easy to use that it has supplanted the refrigerator and ice for all our paraffin sections whether serial or not.

The device, shown in the accompanying figure, is exceedingly simple. A strip of metal about one-eighth inch thick and one inch wide is bent as shown at B and G so as to fit snugly over the casing of the microtome. We happen to use a Spencer (820) microtome so that only two right-angle bends are necessary. At one end of the metal strip a screw (A) permits tightening against the side of the microtome. A common burette clamp (D) is attached to the band of metal and supports a piece of one-fourth inch (outside diameter) copper tubing (C). Copper tubing of this diameter is suitable for paraffin blocks up to approximately one-fourth inch in diameter. For larger paraffin blocks the end of the copper tubing (C') should be spread out, so that the entire block is covered by a flow of gas. A length of rubber tubing (F) leads to the needle valve on a tank of CO₂ gas. The needle valve is supplied free of charge by the company dispensing the CO₂ gas.

The paraffin block (H) is fixed as usual onto the microtome and raised to the highest position possible. The clamp at D is then adjusted so that the nozzle of the copper tube (C') is about one-eighth inch above the paraffin block. The carbon dioxide is now turned on, allowed to flow slowly for about one-half minute, then in a strong stream for about one to two minutes. This cools the knife (K) as well as the block itself. The gas is now shut off and sectioning may begin. If the block appears insufficiently cold at any time, it is only necessary to bring it up to its highest position and again turn on the gas.

We have used this method in cutting adult tissues and such fragile material as early amphibian eggs, using paraffin of varying melting points (45-50°C., 52-54°C., and 56-58°C.). Several persons in this laboratory have been using the same tank of CO₂ (vol. 2400 cu. in.; wt. of gas 59 lb.) for over a year and it is still three-fourths unused, so that we feel the expense for the gas is practically negligible.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

LEONARD, A. BYRON, and LEONARD, ALICE E. A convenient turntable for staining jars. *J. Lab. & Clin. Med.*, 26, 562-3. 1940.

In a circular piece of wood, 1.5 in. thick and 24 in. in diameter, a depression 1 in. deep and 6 in. in diameter is cut on the lower surface to receive the pinion from the transmission gears of an old automobile. The pinion has a bearing on its lower surface which is inset into a block of wood which can be secured to a table. The turntable turns easily on its ball bearings. About 20-22 Coplin jars may be placed on the periphery. The materials cost slightly more than \$1.00. —John T. Myers.

MILLER, ROLAND E., and MOREHEAD, ROBERT P. An inexpensive constant temperature paraffin oven. *J. Lab. & Clin. Med.*, 26, 559-61. 1940.

The oven consists of a wooden box 28 in. long, 17 in. deep and 7 in. high, of 1 in. pine. It is fitted with a permanent top tapering gradually to a height of 14 in. lined with galvanized metal. One side of the box is hinged at its junction with the top. The oven has no bottom, being placed on a sheet of tin on a table. A porcelain tray 26×7 in. serves as a tray for the infiltrating work. Heat is supplied by three 100-watt bulbs set in sockets at the top of the pyramid. The thermostat is a piece of glass tubing 30 in. long and 5 mm. in diameter at one end of which is blown a ball 20 mm. in diameter. At 50 mm. from the ball the tube is bent to form a U. The tube is filled with mercury and inverted except about 10 mm. at the open end. Several drops of chloroform and a small air bubble are forced into the ball, by inversion. Mercury is removed from about 100 mm. of the open end. The tube is placed in the oven with the open end extending thru a hole in the top and the ball at the level of the paraffin. Two wires are forced thru a small cork, one wire placed below the surface of the mercury and the other a few mm. above it. These wires are attached to a Dunco relay. The temperature can be controlled by raising or lowering the wire above the mercury. The surface of the mercury should be cleaned with cotton every 2 months when in service. The oven operates on a 110 v. current and costs only about \$15.00. —John T. Myers.

MICROTECHNIC IN GENERAL

FAVORSKY, M. V. A new method for de-staining slides stained with iron haematoxylin. *Compt. Rend. (Doklady) Acad. Sci. U.S.S.R.*, 26, 493-4. 1940.

After removing slides from iron hematoxylin, rinsing in water and alcohol, the writer decolorizes with a 10% solution of iron alum in glycerin. This solution is placed on slide and mixed with the alcohol. Destaining is very slow, requiring several days, but the process may be watched with an oil immersion objective if necessary.—T. E. Weier.

FAVORSKY, M. V. Role of clove oil in de-staining slides stained with gentian violet after Newton and its possible substitutes. *Compt. Rend. (Doklady) Acad. Sci. U.S.S.R.*, 26, 491-2. 1940.

The following substances, when diluted with alcohol to the thickness of clove oil, may be used instead of clove oil in the Newton gentian violet technic: kedrol, Canada balsam dissolved in xylol, and turpentine which has been thickened by exposure to air for several days.—T. E. Weier.

KRÜGER, FRIEDRICH. Benzylbenzoat—ein geruchloses Intermedium mit günstigen Eigenschaften. *Zool. Anzeiger*, 131, 202-5. 1940.

Benzylbenzoate (a benzoic acid ester of benzyl alcohol) is recommended as a clearing agent after alcohol when the material is to be embedded in paraffin. Its advantages are as follows: refractive index of 1.566; boiling point higher than 150° C. (i.e., in a class with cedar oil and methylbenzoate as to volatility); particularly good for whole preparations, where more volatile fluids that are incompatible with water (e.g., xylene) make the tissue lose elasticity and become brittle; water-clear; colorless; neutral in reaction; not harmful to the most sensitive dyes; miscible with 90% alcohol—therefore tolerant toward water; tissues flexible after long storage; cheaper than methylbenzoate; miscible with collodion and therefore usable, instead of oil of cloves, with collodion; completely miscible with liquid paraffin but scarcely dissolving cold paraffin; not dissolving dry celloidin, but mixing freely with methylbenzoate-celloidin; valuable in clearing Spalteholz preparations. Sections impregnated by the Golgi method have been stored in it without any special precautionary methods and were unchanged after 1½ years.—*Alden B. Dawson*.

PONS, CARLOS A. A substitute for cover glasses. *Amer. J. Clin. Path., Tech. Suppl.*, 4, 189. 1940.

As a substitute for cover glasses the author recommends using grade 1 or heavier cellophane, cut into squares and stored in xylol. To prevent finger marks they should be removed from the xylol by forceps.—*G. H. Chapman*.

DYES AND THEIR BIOLOGICAL USES

CROSSLEY, M. L. The sulfanilamides as chemotherapeutic agents. *J. Amer. Chem. Soc., News Ed.*, 18, 885. 1940.

Certain azo dyes have previously been shown to have bacteriostatic and bactericidal properties. Some of these dyes, namely prontosil and dyes of similar constitution, may be regarded as the forerunners of sulfanilamide, in that these dyes were found to break down in the animal system to give sulfanilamide, and that this product was in all cases the active element. This discovery has opened new possibilities in chemotherapy research and shifted the emphasis from dye structures to simpler molecular systems. Several hundred new sulfanilamide compounds have been synthesized and studied in experimental infections. The evidence does not permit a satisfactory correlation between the structure of the sulfanilamides and their therapeutic effectiveness. However, it is possible to say that when compared in mice with hemolytic streptococcal infection, the majority of the substituted sulfanilamides are less effective than the parent substance given in equal dosages. Any substituent on the nucleus reduces the activity. Substitution of hydrogen on the N¹ or functional group has in many cases given derivatives which are still less active, but a few are more active against hemolytic streptococcal infections and types of infections in which sulfanilamide is not particularly good: e.g. sulfapyridine (2-sulfanilamido-pyridine) has proved effective in pneumococcal infections and sulfathiazole has proved useful for the treatment of staphylococcus infections. Substitution on the N⁴ nitrogen of sulfanilamide has brought about changes which result as a rule in products which are inactive. It is believed that this N⁴ nitrogen is the point at which modification takes place in the animal system, transforming the drug into a harmless substance. The product formed is N⁴-acetyl sulfanilamide. The amount of this product formed varies in individuals and at times in the same individual; therefore, the precise therapeutic effectiveness cannot be predicted. Much work has been done on the problem of the mode of action of sulfanilamide drugs, but all that can be said is that the major effect of the drug is to inhibit the growth of the micro-organisms and this probably makes them more susceptible to destruction by the natural defense mechanism of the host. The relation between the causative agent and the biochemical changes involved in disease is a highly complex problem which must be solved if chemotherapy is ever to make headway.—*A. P. Bradshaw*.

FAVORSKY, M. V. New polyploid-inducing chemicals. *Compt. Rend. (Doklady) Acad. Sci. U.S.S.R.*, 25, 71-4. 1939.

Seedlings of *Hordeum distichum*, var. *nutans* Schubl., were treated with the following substances: aqueous solutions of colchicine, 0.25 and 0.025%; aurantia, 0.01 and 0.001%; anilin, 0.1, 0.05, and 0.001%; trinitrophenol, 0.1 and 0.01%; and phenol, 0.1%. Tribromoanilin was dusted abundantly on the seedlings. The sparingly soluble and poorly volatile substances, diphenylamine, α and β naphthylamine and tribromophenol were applied per se. Iodoform was applied as a gas, 0.01 g. per 200 cc. of air. Nitrobenzene and dichlorodioethyl-sulphide were applied as a 1% solution in vaseline, 1 to 2 g. of the solution being used for each 200 cc. of air. The treatment lasted from 1 to 6 days. It was started either at the outset of germination or when the roots had attained a growth of 1 cm. Aurantia, diphenylamine, and tribromoanilin caused the formation of numerous polyploid cells.—*T. E. Weier*.

FRIEND, D. G. Studies of drugs in tissue metabolism. II. Action of drugs on metabolism of tissue in serum. *Proc. Soc. Exp. Biol. & Med.*, 45, 140-3. 1940.

Methylene blue, at 0.5% concentration, in human serum increased the oxygen uptake of rat liver cells 50%, whereas concentrations of 0.05% and 5% increased it only 20%. The use of phosphate-Ringer's solution instead of serum, or the use of Congo red, rose Bengal-blue (information lacking as to what dye or combination of dyes this may be), brilliant vital red or Evans blue, all used clinically, failed to cause this rise. Following depression of metabolism with chloral hydrate, methylene blue increases metabolism; with sodium barbital, the dye further depresses the metabolism.—*M. S. Marshall*.

GORDON, J. Inhibition of the anaphylactic reaction by Congo red. *J. Path. & Bact.*, 51, 460-3. 1940.

Guinea pigs sensitized with rabbit serum or egg albumin, then repeatedly injected with Congo red before being injected with the shocking dose of the antigen, showed fewer symptoms of anaphylactic shock than controls that had not received the Congo red. It was considered that the Congo red interfered with the entrance of antigen into the cell. The dye itself appeared toxic to a few of the animals.—*S. H. Hutner*.

NORTHEN, H. T. Effects of methylene blue and neutral red on the structural viscosity of protoplasm. *Trans. Amer. Micr. Soc.*, 59, 279-80. 1940.

The effect of methylene blue and neutral red on the viscosity of protoplasm of the fresh water alga, *Spirogyra*, was measured by the displacement of chloroplasts by centrifugation at 680 \times gravity. Methylene blue, 0.0001% in water, did not change the viscosity of the protoplasm within 20 min. but increased it after that up to 210 min.; 0.001% methylene blue did not increase viscosity within 5 min. but did thereafter; 0.001% neutral red in water consistently increased viscosity; 0.01% neutral red decreased viscosity in the measurements made 40 and 90 min. after immersion but increased it at other intervals from 5 to 210 including the measurement at 60 min. (Source of dyes is not indicated nor whether water was distilled).—*Virgene Kavanagh*.

ANIMAL MICROTECHNIC

GINGRICH, WENDELL D. Permanent stained preparations of thick blood films. *J. Parasitology*, 26, Suppl., 20. 1940.

The author presents methods for the preparation of well-stained permanent thick blood films. Recommended procedure: (1) lake and stain dried blood film for 10 min. or more in dilute Giemsa stain (1-3 drops stock solution of unspecified concentration per 1 cc. distilled water), wash momentarily in distilled water and allow to dry; (2) immerse slide in May-Grunwald stain (0.5 g. in 100 cc. absolute methyl alcohol) for 30 sec.; wash momentarily in distilled water and allow to dry. Films of malarial blood, so prepared, on clean, alkali-free slides and mounted in Diaphane are as good after 2 years as at time of preparation.—*Elbert C. Cole*.

PLANT MICROMECHANIC

LEWITSKY, G. A. A cytological study of the progeny of x-rayed *Crepis capillaris* Wallr. *Cytologia*, 11, 1-29. 1940.

Primary root tips were removed and pasted in groups on paper to be fixed and embedded as a unit.—*Virgene W. Kavanagh*.

MICROORGANISMS

COLE, R. M., and DAY, M. F. The use of silver albumose (Protargol) in protozoological technique. *J. Parasitology*, 26, Suppl., 29. 1940.

The authors report the use of the reduced silver method of Bodian (*Anat. Rec.*, 69, 153, 1937) in making cover-glass preparations of protozoa. The technic demonstrates clearly the parabasal apparatus, flagella, undulating membranes and undulating cords of various protozoa. Best results were obtained with material freshly-fixed in Schaudinn's fluid or the formol-acetic No. 2 solution of Bodian.—*Elbert C. Cole*.

DELAPORTE, B. Recherches cytologiques sur les Bactéries et les Cyanophycées. *Rev. Gen. de Botanique*, 51, 615-48; 689-708, 748-68. 1939. 52, 40-8, 75-96, 112-60. 1940.

The Feulgen stain was used extensively in a cytological study of more than 50 species of bacteria and blue-green algae. Smears for Feulgen stains were usually fixed in 95% alcohol for 48 hr., hydrolyzed 10 min. at 60° C., and stained 6 hr. in the decolorized fuchsin. A positive nucleal reaction was obtained with some definite structure in each species studied altho these structures were of different shapes in the various groups. Chromosome-like bodies were found in some of the sulfur bacteria, in some oscillary parasites, and in the blue-green algae, altho the single granule or rod (axial filament) found in the other forms might be considered as a single chromosome.—*Virgene W. Kavanagh*.

ESCHE, PAUL VON DEM. Über auf Endoplatten violett wachsende Stuhlkeime. *Zts. Immunitäts.*, 98, 278-82. 1940.

Stool cultures on Endo agar sometimes yield colonies of intense purple color which is confined to the colony itself and does not diffuse thru the medium. The purple growth is lost after a few subcultures. Microscopic preparations from such purple colonies show cells with red polar granules which can be seen either unstained or when stained with methylene blue. The writer concludes that the color of the colonies is due to the storage of fuchsin in the organism in characteristic polar bodies.—*H. J. Conn*.

KONSCHEGG, TH. Ersparnisse bei der Gram-Färbung. *Münch. Med. Woch.*, 87, 456-7. 1940.

A saturated aqueous picric acid solution is recommended as a mordant in place of the usual iodine solution in the Gram-staining procedure. The saturated solution is used directly when freshly prepared. After a few weeks, this solution is diluted with an equal volume of distilled water before use.

The author also uses a primary staining solution which is prepared as follows: Shake well 3 ml. of anilin oil, 7 ml. of 95% ethanol and 90 ml. of distilled water and filter the mixture thru a wet filter paper. To the filtrate add 2 g. of gentian violet, allow the mixture to stand 24 hr. and then filter.

The staining is carried out as follows: Stain with the above solution, 5 sec.; treat with either saturated or diluted picric acid solution, 5 sec.; decolorize with alcohol; wash with distilled water; counterstain with carbol fuchsin or any other satisfactory stain.—*L. Farber*.

LEVINE, NORMAN D. Changes in the dimensions of *Balantidium* from swine upon cultivation. *Amer. J. Hyg.*, 32, Section C, 1-7. 1940.

The following technic is employed: Examine cecal material from swine just killed, for *Balantidium*. If present, place a small amount of the material in a relatively large amount of Klemmenberg's fixative (saturated picric acid in 2% H₂SO₄). Allow the fixed protozoa to settle. With a pipette, place drops on

slides, apply cover slips and seal with vaseline. Measure 100 individuals with a calibrated 5X ocular and a 4 mm. apochromatic objective. At the same time inoculate 10 tubes of Glaser and Coria's medium (3% horse serum in Ringer's solution with a little powdered rice starch). After cultivation measure the organisms in the same way. The mean length-width ratio becomes smaller, hence *Balantidium suis* may come to resemble *Balantidium coli*.—John T. Myers.

MAYFIELD, CATHERINE R., and GOBER, MAUD. Comparative efficiency of Endo, lithium chloride Endo, desoxycholate-citrate, and bismuth sulfite media for the isolation of *Eberthella typhosa*. *Amer. J. Pub. Health*, 30, 69-76. 1940.

These media were compared for the isolation of typhoid bacteria from feces, typical colonies then being put thru agglutination and fermentation tests. The specimens were inoculated on the media when received, then placed at 20° C. and inoculated again on the second and third days. Plates were incubated for 18 hr. at 37° C. The desoxycholate agar was a dehydrated product of the Baltimore Biological Laboratories; the plain Endo, LiCl Endo, and bismuth sulfite agars were Difco products. Plain Endo and LiCl Endo as prepared by the Mississippi State Board of Health Laboratories were also used. The medium of the latter laboratory consisted of peptone, 10 g., beef extract, 5 g., agar, 30 g., in 1000 cc. water, and adjusted to pH 7.8-8.0. To this was added 1% lactose, and 1 cc. of a 10% Na₂SO₃ solution to which 0.5 cc. of a saturated alcoholic solution of basic fuchsin had been added. The Difco Endo had a slightly different composition. LiCl was added in 0.5% concentration to the plain Endo media. For isolation, LiCl Endo was superior to plain Endo. Comparing LiCl Endo, desoxycholate-citrate and bismuth sulfite, 74% of all the positives (724) were found on the first, 82% on the second, and 83% on the third. Of these 724 positives, 195 were positive only on one medium; 42 on LiCl, 65 on desoxycholate-citrate, and 88 on bismuth sulfite. There were 127 specimens on LiCl, 144 on desoxycholate-citrate, and 246 on bismuth sulfite which were positive on the second and third day's plating that would have been reported negative if only one day's plating had been made. As between specimens examined for diagnosis, release and carriers, there was no marked difference in the efficiency of the three media.—M. W. Jennison.

MOORE, MORRIS. The chorio-allantoic membrane of the developing chick as a medium for the cultivation and histopathologic study of pathogenic fungi. *Amer. J. Path.*, 17, 103-20. 1941.

Fertile hen's eggs were incubated 10-14 days at approximately 38° C. and inoculations made directly into the chorio-allantoic membrane. Successful growth was obtained with 15 pathogenic fungi. Protocols are given for the type of growth and lesions produced by each organism, and the results illustrated by 29 figures.—H. A. Davenport.

MORISON, J. E. Capsulation of haemolytic streptococci in relation to colony formation. *J. Path. & Bact.*, 51, 401-12. 1940.

Exceptionally clear preparations of capsule formation by streptococci can be obtained from colonies grown on sterile No. 300 cellophane (a very thin grade). The cellophane is sterilized by autoclaving between sheets of blotting paper in a separate petri dish, and then dropped aseptically on blood agar. The inoculum is placed upon the cellophane. Capsule staining was most successful when the method of Butt *et al* (*J. Inf. Dis.*, 58, 5, 1936) was followed: Mix a loopful of a dense suspension of organisms with a loopful of 6% glucose. Then mix in a loopful of India ink, and spread like a blood film. Dry without heat. Stain with alkaline methylene blue.

Intact colonies can be stained on the cellophane with most ordinary dyes without objectionable staining of the cellophane.—S. H. Hutner.

RYU, EIHYO. Studies on the simplification of special staining methods of bacteria. I. *Kitasato Arch. Exp. Med.*, 17, 53-7. 1940.

The following simple method for staining the metachromatic granules of the diphtheria bacillus is described: Fix culture with flame or methyl alcohol. Stain

2 or 3 min. without heating in a solution of 5 cc. saturated aqueous $K_2Al_2O_4$ and 1 cc. of saturated alcoholic crystal or methyl violet. Wash well in tap water. The metachromatic granules stain dark purple and the bacterial bodies light purple.

A simple method for staining granules of bacteria is described as follows: Place a loopful of phenol on the slide for a mordant, and smear the culture in it. (For Gram-negative organisms 4-5% aqueous phenol is used, and 7% aqueous phenol for Gram-positive organisms). Dry by flame and fix. Stain 10-30 sec. at room temperature in Ziehl's carbol fuchsin diluted 10 times (Pfeiffer's solution). Wash with water. If a counterstain is desired, stain 30 sec. with Loeffler's methylene blue and wash with water before staining with carbol fuchsin.

Tubercle bacilli may be stained as follows: Place one or two loops of KOH on a slide; mix sputum or culture, and smear; dry and fix. Place a few drops of 0.05% fuchsin on slide, and pass over flame until it boils. After 20-40 sec., wash lightly with water. Stain 30 sec. at room temperature with 2% methylene blue. Wash with water. Examination of sputum of 80 tubercular patients by this method showed 69 positives and 11 negatives. Ziehl-Neelsen's method showed 58 positives and 22 negatives. More organisms per field were demonstrated by the former method than with Ziehl-Neelsen's method.—(From *Biological Abstracts*).

RYU, EIHYO. On a simplified method of staining acid-fast bacteria. II. *Jap. J. Vet. Sci.*, 2, 488-90. 1940.

Smears of sputum or culture are dried and fixed by flaming, then flooded with carbol fuchsin and heated for 30-60 sec. After washing with water, they are counter-stained with 2% aqueous malachite green for 1 min. No decolorization is used.—A. G. Karlson (from *Biological Abstracts*).

RYU, EIHYO. On the Gram-differentiation of bacteria by the simplest method. III. The sulphuric acid method. *Jap. J. Vet. Sci.*, 2, 491-6. 1940.

A half loopful of young agar-slant culture is mixed with half a drop of concentrated H_2SO_4 on a glass slide and mixed thoroly with a platinum loop. The writer claims that Gram-negative bacteria dissolve instantly and the suspension becomes transparent. Gram-positive forms show no change and there remains a turbid suspension.—A. G. Karlson (from *Biological Abstracts*).

SALLE, A. J., SHECHMEISTER, I. L., and McOMIE, W. A. Germicidal efficiency of some medicinal dyes compared to a group of non-dye disinfectants. *Proc. Soc. Exp. Biol. & Med.*, 45, 614-7. 1940.

Fifteen dyes have been tested by the method of comparing toxicity for chick heart tissue and purported bactericidal action. Only the triphenyl methane dyes were found to kill both the typhoid bacillus and staphylococci and of these only brilliant green killed *E. typhosa* under the conditions tested.—M. S. Marshall.

SCHALLOCK, G. Vereinfachter Nachweis von Tuberkelbazillen in histologischen Schnitten durch die Fluoreszenzmethode nach Hagemann. *Münch. Med. Woch.*, 87, 102-3. 1940.

Paraffin sections for demonstration of the tubercle organism are treated as follows: Remove paraffin and treat with absolute alcohol, 95% alcohol, 75% alcohol and distilled water. Stain with 1:1000 auramin, for 15 min. at 37° C.; decolorize with alcoholic HCl (concentration not stated) for 1-3 min. at 37° C.; wash in distilled water; immerse in thiazol yellow 1:1000, 2 sec.; wash in 50% alcohol and in distilled water. Frozen sections are freed from fat before auramin treatment with 95% alcohol or chloroform for a few minutes followed by washing with distilled water.

The tubercle bacilli can be distinguished from the weakly bluish fluorescent background. This method is satisfactory only for material which has been fixed not more than 4-5 days in formalin.

Sections stained with hematoxylin-eosin can be decolorized by immersion for 5-10 min. in a mixture of alcoholic HCl (concentration not given) and 3-5 ml. of 25% H_2SO_4 followed by a wash in distilled water. The decolorized sections can then be stained with auramin etc.—L. Farber.

STAIN TECHNOLOGY



VOLUME 16

JULY, 1941

NUMBER 3

PROGRESS IN THE STANDARDIZATION OF BIOLOGICAL STAINS

INCREASED DEMAND FOR CERTIFIED STAINS

An unexpected increase in the demand for certified stains has occurred during the last year or more. It is believed that this is due very largely to national defense measures and other problems presented by the European war. The situation that has arisen seems to deserve more than passing comment.

It is also a matter of interest at the present time to recall that the Commission on Standardization of Biological Stains is a war baby—or, rather was once a war baby, as it was born following the first world war. The situation at that time was that previous to the war all the biological stains used in this country were imported, and the importation was practically shut off after 1914. The dealers in biological supplies were pretty well stocked up in these items, however, so the shortage was not especially felt until 1917 when the United States began to take part in the war. By this time a dye industry had been established in America, but no special effort had been made to produce the type of dyes needed in biological work. Accordingly, in 1917, when the Government began ordering stains to equip laboratories in hospitals and training camps, these orders were filled very largely with unstandardized dyes of domestic manufacture. Those who worked in any of these laboratories at that period still recall the unsatisfactory results obtained with such dyes.

It has already been mentioned in these pages¹ that the present situation is very different. Practically all the biological stains are now available in reliable quality and this quality is indicated by the Commission certification. It is very clear that the Government now realizes this situation, because practically all of the orders received by the stain companies from purchasing agencies of the Army and Navy are now for certified stains. The Stain Commission has no official status, but such orders show that it now has considerable recognition on the part of the Government.

A few figures can be cited which give a little idea as to how the war

¹Conn, H. J. 1940. Biological stains in time of war. *Stain Techn.* 15, 1-2.

has stimulated the demand for certified stains. In 1934 and 1935 the number of certification labels furnished the manufacturers was approximately 20,000 each year. In 1936 it was approximately 26,000, and essentially the same number in 1937. In 1938 the figure was 29,817; these pre-war figures undoubtedly represent merely a natural increase in the demand. In 1939, however, 36,061 labels were furnished the manufacturers; and as the greatest demand followed the outbreak of hostilities in Europe, it is felt that the war had some influence in causing the increase. In 1940 the figure jumped to 67,543 and approximately two-thirds of this number were furnished the companies during the last half of the year. The figures for the first half of 1941 are not complete yet, but they are approximately the same as for the last half of 1940. It is very interesting that the most striking jump occurred after the national defense measures were begun in the United States.

This increased demand has presented its problems, altho they are distinctly different from those that were faced at the time of the previous war. The demand on the stain manufacturers is proving so great that they are having to prepare batches with unusual haste and sometimes under conditions that do not permit as high quality as usual. Thanks, however, to their coöperation with the Stain Commission, any unsatisfactory stains that result from these conditions are either kept off the market or are at least sold without certification.

A still different type of problem is faced at present in this work and has also resulted from the war. Some users of stains had, until 1940, been continuously employing German products, because they were not able to duplicate their results with stains of American manufacture. This was notably the case in regard to basic fuchsin and Giemsa stain. Now that the German stains are no longer available, such laboratory workers are faced with new problems and several of these have been brought to the attention of the Stain Commission. The majority of these problems are being rapidly solved, and the result undoubtedly is going to be an even greater standardization of the American product. It must be remarked, however, that complaints of this nature ordinarily do not indicate a fault of the American stains so much as the fact that the latter are sometimes adapted to different procedures from those previously imported.

Commission members and stain company employees have given considerable time during the last six months to an investigation of these problems. It seems better, however, that the problems be met and solved in the Commission laboratories than that they be left to face technicians at Army and Navy hospitals and training camps as was the case in 1917.—H. J. CONN.

DYE ADSORPTION BY BACTERIA AT VARYING H-ION CONCENTRATIONS¹

T. M. McCALLA and FRANCIS E. CLARK, *Kansas Agricultural Experiment Station, Manhattan, Kan.*

ABSTRACT.—Adsorption of hydrogen ions and dye cations by washed bacterial cells shows a reciprocal relationship. Apparently, H-ions and crystal violet ions are held by the cell at the same adsorption centers, and the influence of H⁺ on basic dye adsorption is one of direct competition or replacement. The adsorption of H⁺ and acid fuchsin is similar in that an increase is noted as the pH of the suspension is lowered.

Hydrogen ion concentration is known to influence markedly the adsorption of dyestuffs by biological materials (Kindred, 1935; Craig and Wilson, 1937). The relative adsorbability of acid and basic stains at different H-ion concentrations depends upon the isoelectric point of the material employed (Stearns, 1933). At pH values higher than the isoelectric point basic dyes are adsorbed while at lower values acid dyes are adsorbed. This principle, used originally for demonstrating the isoelectric point of gelatin (Loeb, 1922) has been employed for approximate determinations of isoelectric points of bacteria (Tolstoukhov, 1929) as well as of other protoplasmic materials. Levine (1940) found that the staining intensity of protozoan cell structures at controlled pH-values varied not only with different dyes but also with dye or buffer concentrations, and that the isoelectric points obtained by plotting stain intensities for different pH-values also varied. He concluded that the present lack of knowledge of dye-protein combinations prevented determination of true isoelectric points by staining at controlled pH-values.

Since it has been shown that bacteria adsorb cations (McCalla, 1940) and that this phenomenon can be measured quantitatively, the staining of washed bacterial systems at different pH-values appears to offer a means of measuring the quantitative relationships involved in adsorption of dye and of H⁺.

In order to visualize some of the adsorption possibilities in staining procedures, the salient physico-chemical responses of microorganisms are reviewed briefly. Normally, bacteria possess nega-

¹Contribution No. 202, Department of Bacteriology. Coöperative investigations with the Division of Soil Microbiology, Bureau of Plant Industry.

tive charges, and therefore attract positively charged ions. Under favorable cultural conditions, the attracted bases are ions such as K^+ , Ca^{++} , and Mg^{++} , which are essential to biological activity. Such ions fall near the beginning of the Hofmeister lyotropic series and are relatively easy to replace. When bacteria possessing such ions at their adsorption sites are exposed to basic stains, dye cations possessing greater energy of adsorption can be expected to replace the metal cations. If, however, ions of high energy of adsorption, i.e., H^+ , occupy the adsorption sites, the dye cations should meet with strong competition; hence, the number of dye cations adsorbed should show an inverse relationship to the H -ion concentration. When sufficient H^+ is present the charge of the bacterial system becomes neutralized and the isoelectric point is attained. As the system becomes positively charged with the addition of more H^+ , it should become increasingly capable of adsorbing negatively charged ions.

It is the purpose of this investigation to show the quantitative adsorption of acid and basic dyes by bacteria as influenced by H^+ and to attempt an explanation of the phenomenon on the basis of an adsorption exchange process.

EXPERIMENTAL

Crystal violet² and acid fuchsin were made up in 0.01 *M* solutions. Aliquots of the dye solutions were adjusted to pH 0.60, 1.00, 1.50, 1.90, 2.50, 2.90, 3.98, 4.90, 6.95 and 9.10 with HCl or NaOH as needed. All pH determinations were made with the glass electrode. Bacterial cells for dye adsorption were prepared from one-day nutrient agar³ cultures of *Corynebacterium simplex* (Amer. Type Culture Collection No. 6946) and *Bacillus bellus* (Kansas State College stock culture). Surface growth was suspended in distilled water and then washed three times by means of an angle centrifuge. After the final washing, cell suspensions were adjusted to turbidity corresponding to 25 times that of McFarland's nephelometer Tube No. 2. Portions of such cell suspensions were adjusted as nearly as possible to the pH values of the dye solutions.

Employing dye solutions and bacterial suspensions of corresponding pH-values, 1 ml. to 3 ml. of dye solution were added to 5 ml. of bacterial suspension. After thoroly mixing, the bacteria were

²Crystal violet 89% dye-content, and acid fuchsin 60% dye content. Of the former 4.581 g., and of the latter, 9.756 g., were used to make 1000 ml. of aqueous solutions. Dyes used were certified by Commission on Standardization of Biological Stains.

³Agar, 25 g.; peptone, 10 g.; beef extract, 3 g.; NaCl, 5 g.; water to make 1000 ml.; adjusted to neutral reaction with NaOH.

thrown out of the suspension by means of the centrifuge, and the pH value of the remaining solution was determined.

After centrifuging, unadsorbed dye was determined by diluting 1 ml. of the clear fluid with sufficient 2% KH_2PO_4 to assure a readable color range, then comparing in the colorimeter with a standard dye solution similarly treated except for exposure to bacterial cells. Adjusting all unadsorbed dye solutions to the same pH-value by means of the phosphate solution enabled fairly accurate dye measurements. Difference between original dye solution and the unadsorbed, or recovered dye solution, was considered as the amount of dye adsorbed by the bacterial system. The amount of dye adsorbed was expressed as m. mol. (millimolar) per 100 g. bacteria dried at

TABLE 1. THE ADSORPTION OF CRYSTAL VIOLET, ACID FUCHSIN, AND H^+ AT DIFFERENT pH VALUES BY *Corynebacterium simplex* AND *Bacillus bellus* AS M. MOl. PER 100 G. BACTERIA.

| Crystal violet | | | | Acid fuchsin | | | | H^+ | | | |
|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| <i>C. simplex</i> | | <i>B. bellus</i> | | <i>C. simplex</i> | | <i>B. bellus</i> | | <i>C. simplex</i> | | <i>B. bellus</i> | |
| pH of dye-bacteria | m. mol. adsorption | pH of HCl-bacteria | m. mol. adsorption | pH of HCl-bacteria | m. mol. adsorption |
| 0.58 | 19.0 | 0.80 | 0.0 | 0.52 | 15.6 | 0.89 | 21.5 | 0.75 | 119.0 | 1.36 | 77.2 |
| 1.09 | 30.8 | 1.30 | 3.1 | 1.00 | 20.2 | 1.49 | 30.4 | 1.35 | 116.0 | 2.65 | 75.0 |
| 1.62 | 51.3 | 1.82 | 14.1 | 1.50 | 21.0 | 1.90 | 33.2 | 3.80 | 64.5 | 4.20 | 48.6 |
| 1.96 | 68.6 | 2.17 | 24.6 | 2.00 | 17.6 | 2.30 | 33.3 | 6.00 | 22.0 | 5.35 | 25.2 |
| 2.46 | 80.0 | 2.70 | 20.8 | 2.54 | 11.6 | 2.85 | 30.5 | 6.80 | 16.1 | 6.00 | 20.0 |
| 2.84 | 86.6 | 3.35 | 34.8 | 3.15 | 10.2 | 4.00 | 14.2 | — | — | 6.55 | 15.5 |
| 3.65 | 95.0 | 4.20 | 63.3 | 4.40 | 7.4 | 4.95 | 7.4 | — | — | — | — |
| 3.94 | 99.6 | 5.12 | 98.3 | 4.94 | 5.0 | 5.10 | 5.1 | — | — | — | — |
| 5.95 | 109.0 | 6.30 | 106.0 | 6.73 | 4.6 | 7.50 | 1.4 | — | — | — | — |
| 8.40 | 109.0 | 7.79 | 109.0 | — | — | — | — | — | — | — | — |

105° C. Hydrogen ion adsorption was measured by the method of McCalla (1940).

The adsorption of acid and basic dyes and of H^+ by *Corynebacterium simplex* and *Bacillus bellus* is shown in Table 1. Adsorption curves for the latter are shown in figure 1. Altho the results are presented from a single experiment, many similar experiments over a six-months period reveal the data shown to be representative and subject to duplication within narrow limits.

In the pH-range suitable for basic dye adsorption, mixtures of basic dye solution and bacteria revealed pH-values of from 1 to 2 units lower than the values of either the dye or the bacterial components of the system taken separately. Representative decreases

are shown in table 2. Data showing the increased m. mol. adsorption with increased dye concentrations are given in table 3.

DISCUSSION

It is apparent from the data presented that under H-ion concentrations characteristic of normal growing conditions, the micro-organisms studied possessed a high capacity for basic dye adsorption. As the bacterial systems were made more acid, the H^+ adsorp-

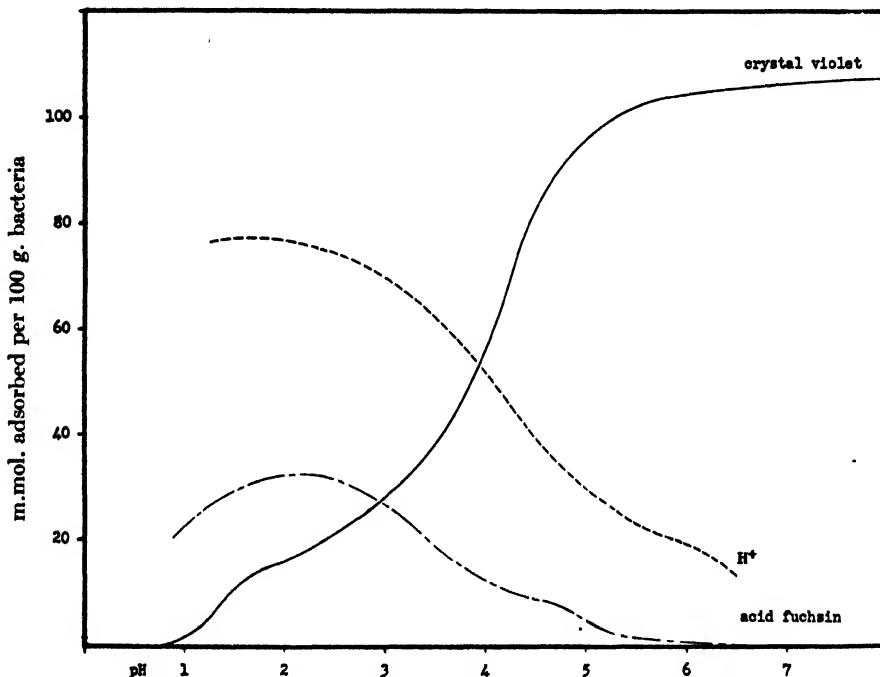


Fig. 1. Adsorption of crystal violet, H^+ , and acid fuchsin by *B. bellus* at different pH values.

tion increased and the ability of the bacterial cells to take up crystal violet decreased; there was a reciprocal relationship between the adsorption of H^+ and of crystal violet. Giesecking and Jenny (1936) have shown that the energy of adsorption of H^+ and methylene blue is approximately the same.

It was observed that in the range of approximately pH 4 to 8, or in the range suitable for basic dye adsorption, mixtures of dye solution and bacteria revealed pH values of from 1 to 2 pH-units lower than the values of either the dye or the bacterial components of the system taken separately. Such sharp drops in pH values in dye-bacteria systems indicate that when crystal violet is adsorbed, it

displaces adsorbed H^+ . In view of such displacement, it is believed that cations are adsorbed at the same sites, and that the adsorption of one ion influences the adsorption of another because of competition for adsorption sites. Such an assumption permits a simple explanation of the action of the factors which have been shown to influence staining intensity, as kind and concentration of dye and nature and concentration of buffer salts. The effective competition of any given dye, as revealed either by staining intensity or by milli-molar adsorp-

TABLE 2. THE DECREASE IN pH VALUE AS INFLUENCED BY CRYSTAL VIOLET ADDITIONS TO *Corynebacterium simplex*.

| pH readings | | | |
|----------------------|--------------|----------------------|------------------------|
| Bacterial suspension | Dye solution | Dye-bacteria mixture | Decrease due to mixing |
| 3.72 | 4.20 | 3.03 | 0.69 |
| 4.14 | 4.20 | 2.97 | 1.07 |
| 5.03 | 5.15 | 3.48 | 1.55 |
| 5.50 | 5.50 | 3.80 | 1.70 |
| 6.50 | 6.15 | 4.80 | 1.70 |
| 8.48 | 8.53 | 6.60 | 1.88 |

TABLE 3. ADSORPTION OF CRYSTAL VIOLET BY *Bacillus bellus* AT DIFFERENT DYE CONCENTRATIONS.

| pH of bacteria | pH of dye | m. mol. of dye added | pH of mixture | Decrease in pH | m. mol. of dye adsorbed |
|----------------|-----------|----------------------|---------------|----------------|-------------------------|
| 7.12 | 6.95 | 45.4 | 6.05 | 0.90 | 45.0 |
| 7.12 | 6.95 | 90.8 | 6.00 | 0.95 | 84.5 |
| 7.12 | 6.95 | 136.3 | 5.86 | 1.09 | 110.0 |
| 7.12 | 6.95 | 181.7 | 5.76 | 1.19 | 115.0 |

tion, should become greater as its concentration increased; this has been found true in the present work. It is also logical that different dye cations should compete with H^+ -ions with varying degrees of success, as do the ions of the Hofmeister series. Likewise, the presence of other ions, from buffer salts or other sources, should be expected to compete with dye cations with varying success according to their nature and concentration.

A relationship is seen between the adsorption of acid fuchsin and of H^+ , in that adsorption for both increases at the lower pH-values. The work of Winslow and associates (1924) on electrophoresis of bacteria has shown that the addition of sufficient H^+ neutralizes the normally negative charge of bacteria. It is probable that with the adsorption of sufficient H^+ , or of other strongly attracted cations, the bacterial cells acquire a positive charge, and that correspondingly,

acid fuchsin or other anions are adsorbed. The influence of H-ion concentration on acid dye adsorption would therefore be the result of changing the charge of the bacterial system, whereas its influence on basic dye adsorption is one of direct competition for adsorption sites.

Data of the nature accumulated indicate that quantitative factors can be established for dye adsorptions, and that recognition of certain fundamental factors may eventually permit regulating the concentration of H⁺ and of other ions sufficiently well to obtain maximum, medium, or minimum adsorption of either acidic or basic dyes by bacteria.

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FROZEN SECTION MICRO-INCINERATION

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ABSTRACT.—A method for micro-incineration of frozen sections is described. Material containing diffusible or soluble salts is cut on the freezing microtome and the sections are placed into xylol and mounted out of xylol onto Corex D slides previously filmed with glycerin-gelatin medium. Material containing non-diffusible or insoluble salts can be fixed in 10% formalin before sectioning. Sections of the fixed material are dehydrated thru 50, 70, and 95% ethyl alcohol and mounted out of absolute alcohol onto Corex D slides previously filmed with glycerin-gelatin medium. After mounting by either procedure the sections are incinerated in an electric furnace and the temperature of incineration is dependent on the type of tissues to be incinerated and the character of the salts present. The method is time saving and when no fixation is required the whole procedure can be carried out in one hour.

The use of micro-incineration has found a varied application in scientific research since its introduction by Raspail. In this laboratory it has been used extensively and many instances have necessitated the development of new technic. It is our purpose to describe such new developments.

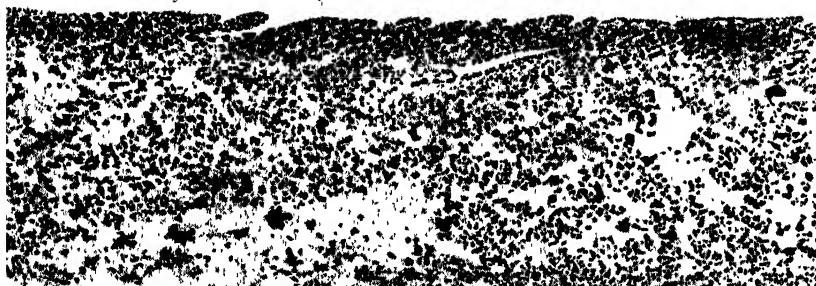
In animal skin research there are inorganic constituents which are of great interest both from a biological and a biochemical viewpoint. In such skins as are potential leather, the presence and condition of the physiological salts influence the skin's inherent quality and also its preservation during "cure". Cure is dehydration by NaCl with the mechanical draining off of some of the albumins and the globulins present.

The amount of physiological salts present is very small, amounting to less than 2% on the native weight of the hide; and as it is so universally distributed throughout the entire substance of the skin, its demonstration requires careful technic. Any appreciable losses by solvents would greatly alter the ash pattern obtained by micro-incineration. Therefore, we have developed for the demonstration of the physiological salts or other readily diffusible salts in skin or in other tissue structures, a simple technic which not only preserves the salts present but also expedites the process.

Fixation. If the salts present are not soluble in formalin and the specimen cannot be sectioned immediately, it is fixed in a 10% formalin-water solution. In the instance where diffusible or soluble salts are present, no fixation is made because the salts will diffuse



A



B

Fig. 1. A. Cross-section of chromed leather stained with hematoxylin. B. Neighboring cross-section after incineration.

out of the specimen into the fixing solution.

Sectioning. Either the fixed or native specimen is sectioned on a freezing microtome using the minimum of water to freeze it. In the case of soluble salts the cut section, approximately 20μ thick, is im-

mediately transferred to xylol and then mounted on a Corex D slide which already has been filmed with glycerin-gelatin medium. After removing the excess medium from around the section, it is flattened out on the slide, by pressing with filter paper, and is ready for incineration. If the salts to be demonstrated are not soluble, the frozen section can be placed into water after cutting. It is then dehydrated in 50, 70, and 95% ethyl alcohol and mounted onto a glycerin-gelatin filmed Corex D slide out of absolute alcohol. Here again the section is flattened out on the slide by pressing with filter paper, and is then ready for incineration.

Incineration. The temperature to be attained for complete incineration depends upon the character of the salts present and the nature of the tissues of the section. This is only determined by experience with the type of sections being incinerated. The following information pertains to the temperatures that give the best results when calf skin or steer hide sections are incinerated.

For demonstration of NaCl and physiological salts in skin we have found that the section can be placed into an electric furnace and heated to 600° C. and then removed to cool. Going much above 600° C. causes dissociation of the salts and gives fusion mixtures instead of crystals. The incinerated section after cooling can be examined immediately. No coverslip is necessary as the ash pattern will not be disrupted unless given a severe jarring.

In our field of investigation, where $\text{Ca}(\text{OH})_2$ is used for removing hair from the skin or hide, we have used incineration as a means of studying the rate of penetration of the lime solution into the skin. Specimens are removed from the lime liquors at given intervals, fixed in 10% formalin, cut into sections on the freezing microtome, and the sections are dehydrated thru alcohols and then mounted onto slides filmed with glycerin-gelatin medium. Because of the insolubility of $\text{Ca}(\text{OH})_2$, the specimens can be fixed in formalin with no noticeable loss of $\text{Ca}(\text{OH})_2$.

Leather tanned in a basic $\text{Cr}_2(\text{SO}_4)_3$ solution, fixed in 10% formalin, and then put thru the procedure mentioned for the limed specimens, gives an incinerated section that shows the chromium as Cr_2O_3 when incinerated at 650° C. For demonstration of the $\text{Cr}_2(\text{SO}_4)_3$ it is necessary to use the xylol technic and incinerate at 650° C.

Examination of Micro-incinerated Sections. The incinerated sections can be examined under bright-field and dark-field illumination. As recommended by Gage,¹ a more complete picture can be obtained

¹Gage, S. H. 1938. Apparatus and Methods for Micro-incineration. *Stain Techn.*, 13, 25-6.

by examining neighboring sections which have been prepared and stained in the usual manner.

To demonstrate some of the pictures obtained by the technic described above, the two photomicrographs in Fig. 1 are shown. The section marked A represents a cross-section of chrome leather stained in the usual manner with hematoxylin while the section marked B shows a neighboring section after incineration. The incinerated section represents a photomicrograph taken in bright field illumination but a similar ash pattern arrangement occurs when the section is examined under dark field illumination.

The application of the frozen section method to the micro-incineration procedure is not only adaptable to animal skin but also works well on certain plant tissues. It is a rapid method and in those cases where no fixation of the specimen is necessary the whole procedure can be carried out in one hour.

THE HISTOCHEMICAL DEMONSTRATION OF HEMOGLOBIN IN BLOOD CELLS AND TISSUE SMEARS

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The demonstration of intracellular hemoglobin in permanent preparations has long been a problem. The affinity of hemoglobin for iron hematoxylin is well known but this stain also colors yolk, chromatin, and other structures and is therefore not a reliable criterion. The presence of hemoglobin has been associated with an acidophil cytoplasm which stains a characteristic color, but a careful

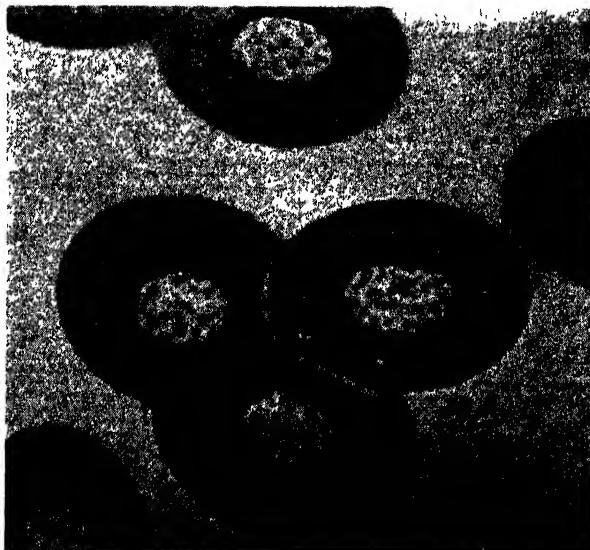


Fig. 1. Adult frog erythrocytes, stained to demonstrate presence of hemoglobin.

inspection of living cells in early hematopoetic or embryological stages demonstrates that hemoglobin is present in the erythrocytes which are quite basophilic. In the course of some research on the blood of embryonic frogs it became desirable to demonstrate the presence of hemoglobin in cells by means of a specific staining reaction.

Lison,¹ Slonimski² and others have described methods for the

¹Lison, L. 1930. Une technique de détection histochimique de l'hémoglobine. Compt. Rend. Soc. Biol., **103**, 86-8.

²Slonimski, P. 1931. Recherches expérimentales sur la genèse de sang chez les amphibiens. Arch. Biol., **42**, 415-77.

demonstration of hemoglobin based on the fact that benzidine in the presence of hemoglobin and water may be oxidized to a brown substance. These methods are not applicable to blood smears. The solution used by Slonimski brings about immediate hemolysis, leaving only the "ghosts" or cell membranes of erythrocytes and the leucocytes with the nucleus stained a rather dark brown. No positive test for hemoglobin is possible upon smeared material with this method. The destruction of cells is due undoubtedly to the presence of acetic acid in the solution.

The procedure for the new method is as follows: An ordinary dried blood smear is flooded with a 1% solution of benzidine in absolute methyl alcohol and allowed to stand for one minute. The benzidine solution is then poured off and replaced with a 25% solution of Superoxol in 70% ethyl alcohol. This is allowed to stand for 1½ minutes, then washed in distilled water for 15 seconds, after which the smear is dried and mounted in neutral Canada balsam. By this method all structures containing hemoglobin are colored a dark brown. Nothing else is stained. The hemoglobin in the living erythrocyte of frog blood is all in the cytoplasm (See Fig. 1). The nucleus is colorless and clear, not dark as maintained by some authors (Slonimski). If it is desirable to study other cells of the blood or the nuclei of the erythrocytes, the preparation may be stained with Wright's stain subsequent to the benzidine procedure with good results. This is useful in the study of such tissue as the spleen where portions of fragmented erythrocytes are phagocytized, or in hematopoietic tissue where red blood cells are being formed. Smears of various organs may be prepared³ by teasing a bit of the organ in cell free blood serum and smearing this on a slide or coverslip. The presence of hemoglobin in any cell is easily demonstrated by this method.

³Issacs, R. 1928. Alteration of tissue cells in the blood stream. Science, 68, 547-8.

AN INEXPENSIVE LAMP FOR THE DISSECTING AND RESEARCH MICROSCOPE¹

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The lamp described herein is inexpensive and serves a dual purpose. It is easy to construct and will provide good illumination for most work with either the dissecting or the research microscope (Fig. 1).

The source of light is a 60-watt bulb with a silvered neck ("neck reflecting"). The use of this type of bulb obviates the need for a reflector in the lamp since the light is concentrated at the end of the

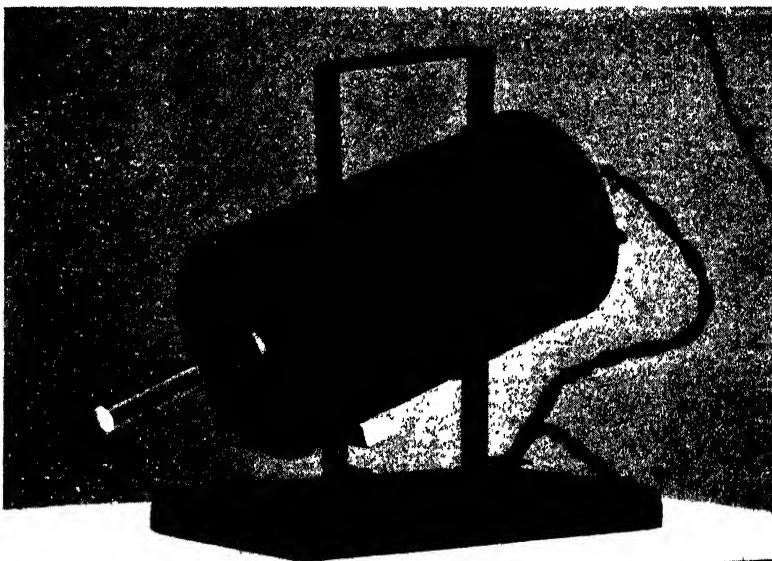


Fig. 1. The finished lamp, assembled.

bulb. A glass rod, 5 inches long by $\frac{7}{16}$ inch in diameter, is used to carry the light from the surface of the bulb to the object to be illuminated. To provide an even field of light, the end surfaces of the rod are ground flat on a carborundum wheel and finished with emery or fine carborundum powder. Probably a rod of lucite could, to ad-

¹Contribution No. 648, Division of Botany and Plant Pathology, Science Service, Dominion Department of Agriculture, Ottawa, Canada.

vantage be substituted for the glass one, altho this has not actually been tried.

The lamp-house is made from a tin can, 4 inches in diameter by $6\frac{1}{2}$ inches deep (a quart motor-oil tin will answer these specifications). The top of the tin is cut away and a hole to take the glass rod is made in the center of the bottom. A collar approximately $1\frac{1}{4}$ inches long of galvanized iron or sheet brass, made to provide a snug sliding fit around the rod, should be soldered in the hole to hold the rod in position. For ventilation, a rectangular opening of about $1 \times 1\frac{1}{4}$ inches is cut lengthwise in the underside of the can near the bottom. In making this opening, the tin is cut along the sides and upper end.

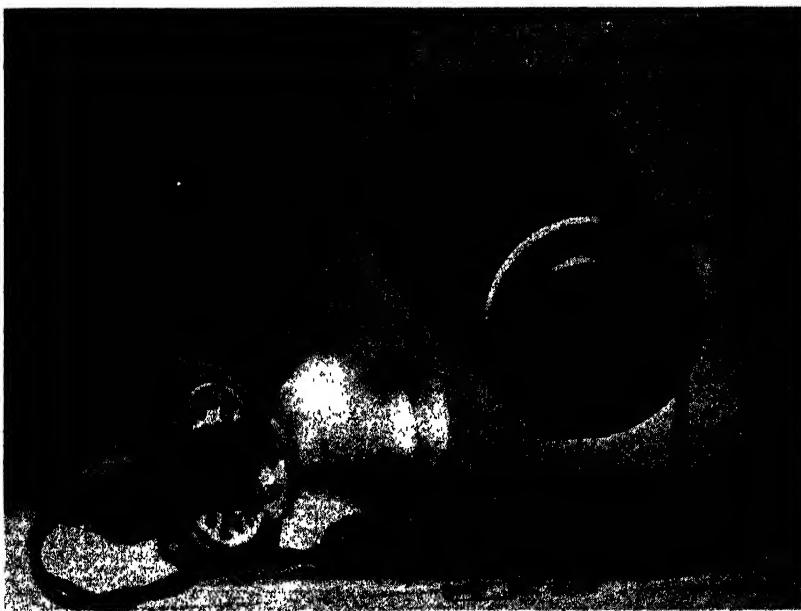


Fig. 2. The lamp with light socket and bulb removed from housing.

This leaves a flap which is bent down about $\frac{1}{4}$ inch and then upward to provide a shield over the opening. The light-socket holder is made from a smaller tin can (one about 3 inches in diameter will serve). The sides are cut away, three strips each about one inch long being left attached at equal distances around the periphery to serve as brackets. A hole, large enough to allow the end of the socket to be screwed thru it, is made at the center of the holder, and small holes for ventilation are punched around this. The socket and bulb are assembled in the holder and this unit is then bolted to the open end of the can with small stove bolts (Fig. 2). An alternative method of constructing this holder would be to screw a shade holder

to the socket and then fit this to the open end with a flange, provision of course being made for ventilation.

The lamp support, which also serves as carrying handle, is made from a strip of strap iron $\frac{1}{8} \times \frac{1}{2}$ inch, which is screwed to a wooden base 5×7 inches in size. The lamp-house is bolted to the support with stove bolts, with a small spring or spring washer on the inside to provide sufficient friction to hold the lamp-house in any desired position.

When using the light with the dissecting microscope, the end of the glass rod is brought as close to the object to be examined as is feasible without its entering the field of view; with the research microscope, the end of the rod may be brought under the stage and rested on the mirror or it may be set back at any convenient distance from it. Some adjustment of light intensity may be obtained by moving the rod in toward, or out from the surface of the bulb. For critical work, light filters and diaphragms may be affixed to the end of the rod.

CLEARING TISSUE WITH MIXTURES OF TRIBUTYL AND TRI-O-CRESYL PHOSPHATES

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ABSTRACT.—Mixtures of tributyl phosphate (Commercial Solvents Corp.) and tri-o-cresyl phosphate (Eastman Kodak Co.), of varying proportions, may be used as immersion fluids for clearing gross specimens. By varying the ratio of the former to that of the latter (measured volumetrically) from 1:130 to 22:110, mixtures having refractive indices varying from 1.555 to 1.534 can be made. A table of mixtures and the index of each is given in this paper. Preparation of specimens consists of skinning, eviscerating, fixing, washing, bleaching (when necessary), dehydrating, and defatting. The proper clearing mixture is determined by the immersion method, which entails microscopic observation of a 50 μ section of tissue to be cleared.

INTRODUCTION

A method is proposed for clearing gross anatomical specimens by immersion in a mixture of two chemical compounds, the refractive index of which can be made the same as that of the tissue which it is desired to clear. The method does not alter the fixed, dehydrated specimen. The cleared material is firm, can be dissected, and can be prepared for histological study. The liquids used are not toxic unless taken internally. Contacts with the skin, such as occasioned in following the method, are not harmful, but the material should be washed off when the work with it is finished.

All methods of this type must be based upon the same principle, and the results achieved can be accounted for by a consideration of the optics involved in the application of that principle. Spalteholz's method¹ is of this kind. His monograph is prolix and interspersed with irrelevant material, and the theoretical treatment is inadequate. Hence a brief discussion of the optics is given here.

OPTICS

In order that an object may be seen clearly and without undue distortion, each interface between media thru which light rays travel

¹Spalteholz, Werner. 1914. Über das Durchsichtigmachen von menschlichen und tierischen Präparaten und seine theoretischen Bedingungen, (Zweite Auflage). Leipzig.

from object to eye must be plane (or spherical with relatively great curvature) and each interface must be of such extent that all light rays from object to eye strike it. Or, if an interface does not meet these requirements, the two adjoining media must have the same refractive index and dispersion. In addition, the light rays must escape absorption by the media and reflection at the interfaces between media of different refractive indices.

Suppose it is desired to clear an animal for demonstration of the skeleton. The animal is killed, skinned, eviscerated, fixed and placed in water. The object (bone) is surrounded by denaturated proteins, fats and water. These are media thru which light must pass to and from the bone. The interfaces, irregular and minute, are countless in number, and the three media have quite different refractive indices. Even if the bone could be illuminated, it could not be seen because light rays coming from it would undergo deviation and reflection at the myriad interfaces between the media of different refractive indices.

In order to convert such a system into one in which the bone becomes clearly visible, the following steps are taken: bleaching of pigments (if necessary), dehydrating, defatting and replacing the final solvent with a fluid whose refractive index is the same as that of the principal tissue which surrounds the bone, that is, striated muscle. Light rays leaving the bone are confronted by innumerable, tiny interfaces as in the previous case, but now the media have the same refractive indices. (Practically, it is not necessary to consider dispersion.) Hence the light rays undergo neither deviation nor reflection² until they meet the interface between the liquid and air. Although the liquid and air have very different refractive indices, the visualization of the object is not spoiled because the interface is plane. The bone itself is not transparent because its inorganic material has a refractive index different from that of the liquid even tho its organic material has nearly the same index. Cartilage and nervous tissue are also visible in a specimen cleared as outlined above.

TECHNICAL PROCEDURE

Preliminary treatment of specimens. If a whole animal is used to demonstrate bone and cartilage, it should be bled thoroly, skinned and eviscerated. The refractive indices of skin and muscle are

²That the deviation is zero when the refractive indices are equal follows directly from Snell's law. It can be seen that the reflection coefficient, R, which for normal incidence is $\left(\frac{n_2 - n_1}{n_2 + n_1}\right)^2$ becomes zero when the refractive indices are equal.

different, hence it is impossible to clear both with the same liquid, altho both may be partially cleared by employing a liquid of intermediate refractive index. Evisceration eliminates a large amount of blood and pigment, and also food in the gut.

Fixation. A solution of commercial formalin (10 cc. in 90 cc. water) or a mixture of 5 cc. formalin in 65 cc. 95% ethyl alcohol, and 30 cc. water, is recommended. Other fixatives can be used. The fixative will decalcify if acid.

Washing. After fixation the tissue should be washed sufficiently to remove all the fixative.

Bleaching. Hydrogen peroxide, U. S. P. (3% H_2O_2), is used without dilution. If decalcification is to be prevented, the solution should be neutralized before use. An adult rat will require a week or more and probably one change of solution, while small bits of tissue should bleach in a day. The bubbles formed in the tissue can be removed by placing the specimen in water in a vacuum jar and evacuating. Any bubbles which remain after clearing can be removed by again evacuating (since the clearing fluid is not volatile) or by drawing the bubbles out with a syringe and hypodermic needle.

Washing. The tissue should be washed sufficiently to remove all H_2O_2 .

Dehydration. Either acetone or dioxan can be used. A half-and-half mixture of water and dehydrating agent is followed by two changes of pure dehydrating agent, or more gradual transitions from water to dehydrating agent may be made. The dehydrating agent also removes fat. This is very necessary inasmuch as fats have a lower refractive index than proteins. Complete removal of water and of fat from an adult rat requires three or four days in each change of the pure acetone or dioxan.

Determination of the refractive index of the particular tissue to be cleared. The refractive index of a bit of tissue can be determined to the third decimal place by the immersion method. This is a method commonly employed by mineralogists.³ The significance of this degree of accuracy in connection with the clearing of tissues is questionable because closely adjoining parts of a given tissue may vary slightly in refractive index, the refractive index of the liquid medium used changes with the temperature, and the dispersions of the liquid and of the tissues are not the same. The best medium is the one which has the same refractive index as most of the particular tissue to be cleared, at the temperature at which the cleared specimen is most likely to be observed.

³See Winchell, Alexander N. 1937. Elements of Optical Mineralogy, 5th Ed. (Part I.) John Wiley and Sons, Inc., New York.

A series of liquids of different refractive indices is prepared by mixing tri-o-cresyl phosphate and tributyl phosphate (Table 1). This series covers the range of refractive indices likely to be met in tissues.

After the specimen has been completely dehydrated and defatted, a small portion of the tissue to be cleared is cut from the specimen and imbedded in paraffin by the usual procedure. The tissue may be put directly into melted paraffin from either dioxan or acetone. Sections 50 μ thick are cut. The sections at this thickness curl, and it is necessary to float them on warm water to flatten them. They are picked up from the surface of the water on clean microscope slides. No adhesive should be used. Only one slide carrying one section is required, but two slides facilitate the determination.

When the sections are completely dry, the slides are immersed in xylene to remove the paraffin. When the slide is removed from the xylene, the section is washed with several drops of xylene from a medicine dropper, and the excess xylene drained from the slide. Before the section dries, one drop of one of the refractive index liquids is placed on the section. The slide is tilted in different directions so that the liquid washes over the entire section. The drop is drained off with the aid of a blotter or paper towel, and the procedure repeated. The third drop is allowed to remain on the section. The slide is placed under the 16 mm. objective of a microscope. Normal illumination is used. The condenser should be lowered somewhat, and the diaphragm closed so that the field is rather dark. The section is brought into sharp focus. The objective is then raised very slightly with the fine adjustment. A narrow, bright line called the Becke line will be seen along every edge between tissue and liquid. If the liquid has a higher refractive index than the tissue, the Becke line will appear to move from the edge out into the liquid when the objective is raised. If the liquid has a lower refractive index than the tissue, the Becke line will appear to move into the tissue. The Becke line will be strong if the difference between the refractive indices of tissue and liquid is great, and will be scarcely noticeable if the difference is slight. If the first liquid tried is high, one having a lower index is next used. The intensity of the Becke line in the first instance will serve to indicate how much lower the second liquid should be. The first liquid is washed off with xylene from a medicine dropper, and the second liquid is applied just as the first one was.

In some cases it might be quite difficult to observe the Becke line when the refractive index of the liquid is very close to that of the tissue. This is especially true if monochromatic light is not used. In many instances the same kind of tissue in one section may vary

enough in refractive index so that the refractive index of a certain liquid may be at once higher than, lower than, or the same as that of the tissue in neighboring parts of the section as determined by the Becke line. At such times, the tissue should be assigned the refractive index of the liquid in which it displays the greatest average transparency. At the start of the procedure, while the section is still immersed in xylene, it should be carefully studied as to gross extent and as to distribution of different types of tissue. This step is indispensable. For example, a section consisting principally of striated muscle is being examined to determine the index of that tissue. When a liquid is used which has a refractive index quite close to that of the muscle, it is not possible to recognize that tissue. If the Becke line is observed, it is imperative to know whether the line comes from muscle or from adjacent collagenous connective tissue. Much of the muscle tissue may be rendered entirely invisible, and hence the extent of the section must be known in order to estimate its average transparency.

When the refractive index of the tissue has been determined thus, the entire specimen is immersed in a mixture similar to the one in the refractive index series from which the tissue index has been assigned. The specimen is transferred to the clearing mixture directly from the last change of dioxan or acetone. The container should be covered only with cheese-cloth to exclude dust until the volatile solvent has evaporated. Evacuating the preparation will hasten the removal of the solvent. If the pressure becomes too low, the tributyl phosphate may evaporate slowly but this situation can be readily recognized because the tributyl phosphate condenses on the inside of the vacuum jar.

Preparation of the refractive index series of liquids. The immersion liquids are relatively inexpensive and are prepared by mixing tri-o-cresyl phosphate and tributyl phosphate in various proportions. The tri-o-cresyl phosphate used is the technical grade sold by Eastman Kodak Company, Rochester, New York. It has a boiling point of 265° C. at 20 mm. of mercury, and a density of 1.165 g. per cc. at room temperatures. It has a very faint yellow tint. Tributyl phosphate is sold by Commercial Solvents Corporation, Terre Haute, Indiana. It boils at 178° C. at 27 mm. of mercury. Its density at room temperatures is 0.970 g. per cc. The compound is colorless. The refractive index of tri-o-cresyl phosphate is n_D^{22} equals 1.5560; of tributyl phosphate, n_D^{22} equals 1.4229. The boiling point of each of these liquids is exceedingly high. The vapor pressure of each at room temperature is practically zero. Hence, no alteration of the composition of the mixtures occurs thru evaporation at room tem-

perature. They will not vary enough among lots to affect the refractive indices of the mixtures in the third decimal place. The change of refractive index with temperature is about the same for all the mixtures. For example, n_D of liquid 17 is, at 20° C., 1.5397; at 22° C., 1.5389; at 25° C., 1.5377.

TABLE 1. MIXTURES OF TRI-O-CRESYL PHOSPHATE AND TRIBUTYL PHOSPHATE FOR USE AS IMMERSION LIQUIDS.

| Number | C. C. Tributyl Phosphate | C. C. Tri-o-cresyl Phosphate | n_D^{22} desired | n_D^{22} determined on Abbé refractometer |
|--------|--------------------------------|------------------------------------|--------------------|---------------------------------------------------|
| 1 | 1 | 130 | 1.5550 | 1.5550 |
| 2 | 2 | 130 | 1.5540 | 1.5540 |
| 3 | 3 | 130 | 1.5530 | 1.5530 |
| 4 | 4 | 120 | 1.5520 | 1.5519 |
| 5 | 5 | 120 | 1.5510 | 1.5509 |
| 6 | 6 | 120 | 1.5500 | 1.5500 |
| 7 | 7 | 120 | 1.5490 | 1.5490 |
| 8 | 8 | 120 | 1.5480 | 1.5480 |
| 9 | 9 | 120 | 1.5470 | 1.5471 |
| 10 | 10 | 115 | 1.5460 | 1.5459 |
| 11 | 11 | 115 | 1.5450 | 1.5449 |
| 12 | 12 | 115 | 1.5440 | 1.5440 |
| 13 | 13 | 115 | 1.5430 | 1.5430 |
| 14 | 14 | 115 | 1.5420 | 1.5420 |
| 15 | 15 | 110 | 1.5410 | 1.5408 |
| 16 | 16 | 110 | 1.5400 | 1.5399 |
| 17 | 17 | 110 | 1.5390 | 1.5389 |
| 18 | 18 | 110 | 1.5380 | 1.5379 |
| 19 | 19 | 110 | 1.5370 | 1.5371 |
| 20 | 20 | 110 | 1.5360 | 1.5361 |
| 21 | 21 | 110 | 1.5350 | 1.5352 |
| 22 | 22 | 110 | 1.5340 | 1.5343 |

In the accompanying table, 22 mixtures are listed ranging in refractive index from 1.5550 to 1.5343. Of this number, all may not be of use, but all are included for the sake of completeness. Only liquids 12 to 22 need be prepared unless others prove to be necessary. Refractive indices were determined with an Abbé refractometer. The mixtures were prepared so that each differs from its neighbors in the series by one in the third decimal place. The indices correct to the fourth decimal place, as determined with the Abbé, are also given in the table. The refractive index liquids used for testing tissues are conveniently prepared in quantities one-tenth those given in the table. Glass syringes serve well for measuring the two liquids in making up the series. The refractive index liquids should be kept in clean glass containers, and care should be taken to prevent contamination.

SUPPLEMENTARY NOTES

If accurate determinations of the refractive indices of tissues are desired, monochromatic light (the same as that for which the indices

of the liquids were determined) should be used. If, however, it is desired simply to find the liquid in which the tissue is most transparent, white light is satisfactory. It is not always necessary to make index determinations once they are learned for a given type of tissue; for example, the deep back musculature of young or adult rats has an index of 1.537 ± 0.001 .

Rats, ground squirrels, bats, moles, fetal rabbits, and human fetuses have been cleared, and liquid 19 (Table 1) has served as a good clearing medium for all, altho it was not the best for some specimens.

Museum jars containing the clearing liquid can be sealed with glue, but may be merely covered because the liquid is not volatile.

The advice of Dr. R. C. Emmons, Dr. J. E. Mack and especially Dr. H. W. Mossman has been of great aid in the execution of this work.

STAINING THE NUCLEOLUS

C. S. SEMMENS AND P. N. BHADURI, *Kings College, University of London, Bristol, England*

About two years ago, the authors¹ described a cytological stain combining leuco-fuchsin and light green. The original technic has caused some difficulty, because the use of aqueous Na_2CO_3 in mordanting causes stripping of serial sections from the slides, unless properly hardened. While recent developments of the technic have been mainly concerned with the production of efficient and rapid smear methods (Bhaduri²) the use of an alcoholic mordant now does away with the need for any special treatment to avoid the stripping difficulty with serial sections.

The schedule given below can be recommended with confidence to those who wish to try the nucleolar staining method on microtome sections of either plant or animal material mounted on slides in the usual way. As previously mentioned, chrom-formalin fixatives give very good results with most plant materials used for mitosis; meiotic material may be fixed in Semmens' sodium diuranate mixture (Bhaduri and Semmens, 1940).³ Most fixatives will, however, give good results if before hydrolysis the material is left in 1% chromic acid solution for a few hours; it should then be thoroly washed in water to remove all traces of chromic acid and left for about four hours or overnight in 75% alcohol.

STAINING SCHEDULE

1. Slides with wax ribbons are run down to water in the usual way except that it is best to leave them in the 75% alcohol grade for at least two hours.
2. Wash with warm water and leave standing in warm water for about five minutes, transfer to 12% HCl at 60° C. and keep at this temperature to hydrolyze for the optimum period, (20 seconds is a good average time to try for new or unknown plant material).
3. Remove the staining jar from the oven or heating apparatus used and immerse it in a vessel of cold water in which cooling of the slides may take place. When cold pour off the used acid and rinse

¹SEMMENS, C. S., and BHADURI, P. N. 1939. A new method for differential staining of the nucleolus. *Stain Techn.*, **14**, 1-5.

²BHADURI, P. N. 1938. Root tip smear technique and the differential staining of the nucleolus. *J. Roy. Micr. Soc.*, **58**, 120-4. 1940. Improved smear methods for rapid double staining. *J. Roy. Micr. Soc.*, **60**, 1-7.

³BHADURI, P. N., and SEMMENS, C. S. 1940. Advantages of uranium fixation in modern cytological technique. *Nature*, **146**, 100.

the slides with fresh cold *N* HCl, pour this off and cover them with decolorized fuchsin. Leave in this for 3-4 hours or overnight, as convenient.

4. Pour off the dye and cover the slides with bleaching solution, using two changes of this with half an hour in each.

5. Rinse in distilled water, in 50% alcohol, then 70% alcohol.

6. Place slides in mordant solution, consisting of 80% alcohol saturated with Na_2CO_3 ; leave in this mordant for at least one hour.

7. Pour off the mordant solution and rinse the slides with 80% alcohol to remove all traces of mordant; rinse quickly in 95% alcohol and then place 20-25 minutes in a light green solution prepared as follows: after filtering a saturated alcoholic solution of light green S. F. Y. (C. I. 670), add 2-3 drops of pure colorless anilin oil per 100 cc. of dye.

8. Drain off the dye and rinse the slide, for preliminary treatment, in a differentiating solution consisting of: Na_2CO_3 , saturated solution in 80% alcohol, 10 cc.; 80% alcohol, 90 cc. Continue the differentiation in 95% alcohol, examining under the microscope to watch progress. If necessary, return to the differentiating solution for further treatment and then back to the 95% alcohol. In this way differentiation can be carefully controlled until the green is cleared from everything but the nucleoli. The last traces of green may of course be removed from the cytoplasm in the process of dehydration.

9. Dehydrate in two or three changes of absolute alcohol.

10. Clear in alcohol-xylol (50-50), xylol-alcohol (75-25) followed by pure xylol.

11. Mount in "Sira" mountant or some similar neutral medium.

As frequently pointed out, it is necessary to determine by experiment the best period and temperature for hydrolysis with any new material and a clear-cut Feulgen reaction is only to be expected if this is done. Hydrolysis of material on slides is best carried out in a porcelain Coplin jar; the thick glass of the ordinary staining jars will not stand the necessary heating and cooling treatment.

Material that has been fixed in osmium fixatives can usually be stained by the following procedure: Bleach as usual in 3 parts of 80% alcohol to 1 part of 20 vol. hydrogen peroxide. Transfer to 70% alcohol for 1-3 hours to harden, then run down to water. Mordant in 1% chromic acid as mentioned earlier, then carry on with the above schedule.

Instructions for the preparation of decolorized fuchsin and the bleaching solution may be found in previous numbers of Stain Technology.

CHLORAZOL BLACK E, A SIMPLE CONNECTIVE TISSUE STAIN

NORMAN D. LEVINE¹ and C. C. MORRILL,¹ *Animal Pathology Laboratory, University of Illinois, Urbana, Ill.*

Cannon,² Darrow³ and Nebel⁴ have reported that chlorazol black E is of value as a biological stain. The dye, according to Darrow, is



Fig. 1



Fig. 2

Slightly diagonal section of an artery (125 \times) stained with chlorazol black E (Fig. 1) and with hematoxylin and eosin (Fig. 2). Note the contrast in appearance of elastic fibers.

synonymous with Erie black GXOO (National Aniline and Chemical Co.), pontamine black E (I. E. du Pont de Nemours Corp.) and chlorazol black E (British Dyestuffs Corp.).

Darrow recommended staining in a 1% solution of the dye for 5 to

¹Assigned by the State Department of Agriculture to the Animal Pathology and Hygiene Laboratory to assist in diagnosis and research.

²Cannon, H. J. A new biological stain for general purposes. *Nature*, **139**, 549. 1937.

³Darrow, M. A. A simple staining method for histology and cytology. *Stain Techn.*, **15**, 67. 1940.

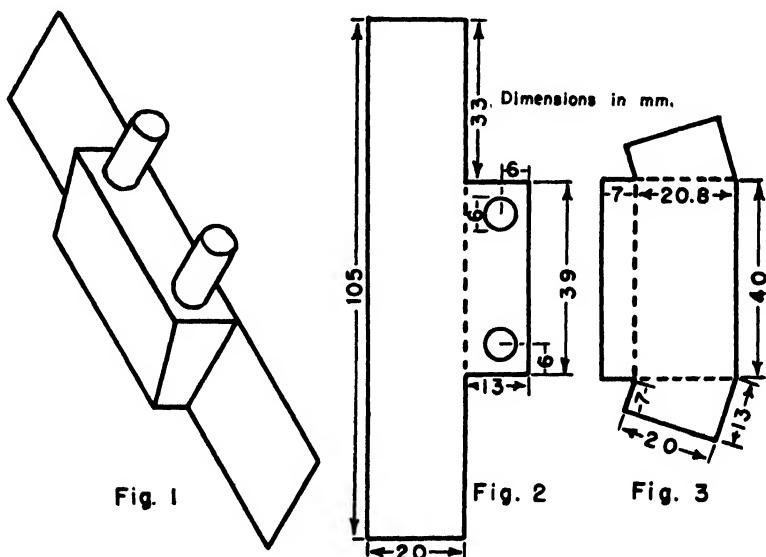
⁴Nebel, B. R. Chlorazol black E as an aceto-carmine auxiliary stain. *Stain Techn.*, **15**, 69. 1940.

10 minutes, and then dehydrating, clearing and mounting, while Cannon suggested a saturated solution in 70% alcohol. Since a 0.5% solution in 70% alcohol of the sample used by the writers was saturated, there appeared to be no point in using a 1% solution. A staining period of 30 minutes was considered preferable to the shorter time. Neither mordanting nor destaining is necessary.

The writers have confirmed Darrow's findings that chlorazol black E gives sharp differentiation in histological and cytological preparations, and stains different tissue elements black, green or yellowish green. It is noteworthy that the elastic fibers of connective tissue stain an intense black (Fig. 1, 2). Since many of the staining procedures used in the study of these elements are relatively complicated and time-consuming, it is felt that the simplicity of the staining technic with chlorazol black E should recommend it to histologists for such studies.

NOTES ON TECHNIC

TEMPERATURE CONTROL FOR MICROTOME KNIVES.—Temperature control during paraffin sectioning is readily obtained if a safety razor blade holder with provision for water circulation is used. With a microtome knife, however, the problem is more complicated. A device described by Cooper and MacKnight¹ provides for chilling but does not seem quite as well suited for warming the knife—a procedure which is often necessary when thick sections are cut. The device shown in Figure 1 may be used to chill or warm a microtome knife by means of water circulation. The essential features are that it presents a large flat surface for close contact and heat exchange with the knife, and is very readily attached.



Figs. 1-3. Device for chilling microtome knife.

The water-jacket consists of two pieces of $\frac{1}{2}$ inch sheet copper soldered together and two nipples of $\frac{3}{8}$ or $\frac{1}{4}$ inch copper or brass tubing for the attachment of rubber hoses. The design of the component pieces is shown in Figs. 2 and 3. Dimensions (which are indicated in millimeters) may have to be altered to fit various sizes

¹Cooper, K. W., and MacKnight, R. H. Cooling device for the microtome. *Stain Techn.*, 12, 25-7. 1937.

and types of knives and knife-holders. If a hollow ground knife is used, the contact surface of the water-jacket must be curved to fit.

To attach the water-jacket to the knife, the ends of the contact plate are interposed between the clamp screws and the knife. Sufficient space should be left between the knife edge and the top of the water-jacket to accommodate at least one section; otherwise it may be difficult to start a ribbon.—JOHN W. DUFFIELD. Northeastern Forest Experiment Station, New Haven, Conn.

A USEFUL EMBEDDING TECHNIC—The following simple but successful method for embedding insect or other tissue in paraffin for microscopical serial section has been developed. It eliminates most of the difficulties that are experienced in embedding. It differs from the usual methods in that very hot paraffin is employed and ice cubes are used to cool the paraffin in the embedding boats. The steps in the process are as follows:

1. Kill, fix, dehydrate, and infiltrate as usual.
2. Heat the embedding paraffin and the ends of a pair of forceps in dishes over boiling water to 180° F. or above.
3. Fill an embedding boat with the hot paraffin and place it on an ice cube to cool. Two or more boats may be filled at the same time, as experience allows.
4. When the lower portion of the paraffin is completely opaque, lift the tissue by means of the hot forceps from the infiltration chamber to a dish of hot paraffin for an instant, or until the cool paraffin around the tissue is melted, and then transfer it to the embedding boat.
5. Orient and allow to cool.

The success of the method lies in the fact that the paraffin is cooled much more rapidly from the bottom than it is from the top. This allows the placing of the tissue well up in the block, exactly at the freezing point of the paraffin, without the danger of layering. The hot paraffin above and the hot forceps allow leisurely orientation of the tissue without the danger of introducing air bubbles. The rapid cooling aids in producing small paraffin crystals. No trouble has been experienced with cupping or treeing. The use of thin cotton gloves may aid some people in handling the heated forceps.—C. E. WOODWORTH, U. S. Dept. Agriculture, Walla Walla, Wash.

A METHOD FOR NUMBERING SERIAL CELLOIDIN SECTIONS.—A method for marking celloidin sections has been developed to permit staining large numbers of serial sections at one time without first mounting them on slides. This involves a marking substance which, unlike India ink and similar substances, is permanent except in solvents of celloidin.

Sections are cut and placed chronologically between slips of paper in the usual manner. An "ink" is prepared by thoroly mixing equal parts of 20% nitrocellulose (Hercules Powder Co., RS $\frac{1}{2}$ sec.) in amyl acetate¹ and black oil color (e. g. Martini Studio Oil Color, Ivory Black). One corner of each section is blotted and then numbered by means of a small camel's hair brush. Before returning the sections to the alcohol, the ink is "fixed" with a drop of chloroform applied with a second brush. Numbers applied in this way become an integral part of the celloidin around the section and do not wash off. The substance here described is more viscous than those usually used, but with a little practice it can be applied as easily and rapidly as any other numbering material.—JOHN MEACHAM HAMILTON, Laboratory of Neurophysiology, Yale University School of Medicine, New Haven, Connecticut.

A FORMALIN-PHENOL-THIONIN STAIN FOR NERVOUS TISSUE.—Fresh nervous tissue or nervous tissue treated according to the Marchi technic (Swank modification) stains rapidly (1–5 minutes) and well with the usual aqueous phenol-thionin procedure.

If, however, it is fixed by perfusion with 10% formalin, and subsequently stored in this reagent, it stains very lightly in 1% aqueous phenol-thionin, even tho applied for several hours or overnight. To hasten and intensify the staining, several modifications have been tried and the following procedure adopted as most desirable.

1. Wash nitrocellulose sections in distilled water.
2. Stain 15–30 minutes at 50° C. in the following mixture: 25 cc. 0.5% aqueous thionin (National Aniline Co., C. I. 920) plus 2 drops of phenol (liquefied) and 2 drops of formaldehyde (34–38% U. S. P.).
3. Rinse in distilled water.

¹Nitrocellulose is sold in a dehydrated form consisting of about 10 parts nitrocellulose to 7 parts absolute alcohol. The 20% solution mentioned above is made by adding 20 g. of nitrocellulose-alcohol mixture to 80 cc. amyl acetate.

4. Wash well in 70% ethyl alcohol.
5. Place in 95% alcohol 2-4 minutes.
6. Absolute alcohol plus 12-15% of U. S. P. chloroform.
7. Clear and differentiate sections individually 1-10 minutes in equal parts of: oil of origanum, cretic; oil of bergamot, f. b. extra fine; phenol. Avoid creosote!
8. Place on clean glass slide with forceps. Low volatility of clearing fluid allows adequate time to orient serial sections. If sections become too dry or stick to the slide in improper position, dampen with a brush dipped in the clearing fluid.
9. Roll sections flat with a camel hair brush. Blot dry with bibulous paper. Complete drying and flattening process with a silk cloth wrapped around index finger.
10. Wipe around sections with a cloth moistened in xylol to remove oil droplets. Care must be exercised that the sections do not become so dry that they show silvery patches when viewed from the reverse side of the slide. Slight dampening with clearing fluid will correct this.
11. Remove any lint or dirt with a clean, dry, camel hair brush.
12. Mount in damar, clarite or balsam.

Sections are more easily stained if not stored in alcohol for more than a week. U. S. P. formaldehyde was found to give less background staining than that of reagent quality. Altho staining can be accomplished without phenol, it probably retards fading of the sections. Several hours (e.g., overnight) in 70% alcohol does no harm if sections are deeply stained. If desired, sections may be left overnight in absolute alcohol plus chloroform. The origanum-bergamot-phenol clearing fluid may be modified by adding a small amount of methyl salicylate or amy1 acetate to make sections more pliable. Oil of lemon, Californian U. S. P. (XI), may be used as a differentiating fluid prior to clearing. Synthetic oil of bergamot may be used instead of the more expensive f.b. extra fine grade. Coverslip weights should be used if thick sections are mounted. Large infant brain sections, stained in formalin-phenol-thionin or by Weil's technic for myelin, are also easily mounted on large slides or between lantern slide cover slips in this manner. Nitrocellulose used in developing the above technic was procured from Charles Cooper, Manufacturing Chemists, New York. GEORGE C. GRANT, Division of Anatomy, University of Tennessee College of Medicine, Memphis.

STAIN FADING IN VARIOUS HISTOLOGIC MOUNTING MEDIA.—Sets of five sections each of monkey pancreas, adrenal, spleen, kidney, and heart were stained in Weigert's iron chloride hematoxylin and Van Gieson's picrofuchsin, using 0.1 g. acid fuchsin per 100 cc. saturated aqueous picric acid and another similar series with our buffered Romanowsky stain at pH 4.2.

One set of sections with each of the two stains was mounted in neutral Canada balsam in xylol, one set in Curtis' salicylic acid balsam, one set in 60% clarite in xylol, one set in 60% clarite X in xylol and the last set in heavy liquid petrolatum U. S. P. ($N\Delta = 1.483$) and sealed with pyroxylin cement.

The pancreas and adrenal sections were exposed continuously under a 100 watt electric light at a distance of 22-25 cm. in a box painted inside with aluminum paint and on an aluminum tray. The others were left on a north window sill in diffuse daylight for 6 months.

The blue component of the Romanowsky stain showed appreciable fading in salicylic balsam in 90 minutes and was entirely gone in 24 hours under the lamp. In 5 days, fading was moderate in neutral balsam, and in 10 days quite marked. At 10 days both clarite and clarite X showed slight fading. After 3 months under the lamp and after 6 months in daylight, the salicylic and the neutral balsam showed complete fading of the blue component, clarite X quite marked fading, clarite moderate fading, and the liquid petrolatum practically no change.

The iron-hematoxylin picrofuchsin stains, after 6 months in daylight or 3 months under the electric light, showed red collagen with clarite and clarite X, pink collagen with salicylic acid balsam and liquid petrolatum, and completely unstained collagen with neutral balsam. The nuclear staining showed a slight loss of intensity in the salicylic balsam and was unchanged in the other media.

Of the five, clarite appears to be the most useful mounting medium for the two stains. Liquid petrolatum, while showing nearly perfect preservation of Romanowsky staining, requires cementing of cover glasses, and has a considerable tendency to leak, creating an oily film not only on the adjacent part of the slide, but also over the margins of the cover glass for several millimeters.—R. D. LILLIE, Division of Pathology, National Institute of Health, Washington, D. C.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

DEROW, HARRY A. An inexpensive polarizing device for microscopes. *J. Lab. & Clin. Med.*, 26, 694-5. 1941.

A search should always be made with a polarizing microscope for doubly refractile bodies in a urinary sediment in a case of massive albuminuria. An inexpensive type may be made as follows: Unscrew the outer lens mount of the ocular and apply Canada balsam to the rim on its under surface; attach an appropriate circle of polaroid film to serve as the analyzer; slip a wide circle of polaroid film into the slot under the condenser and leave it permanently to serve as a polarizer. Polaroid film may be purchased from The Polaroid Co., 285 Columbus Avenue, Boston, at \$4.00 per 4 inch square. The polarizer and analyzer should be set at right angles by rotating the eye piece until there is a reddish-purplish-black field; then doubly refractile bodies appear as Maltese crosses. Such bodies occur in the nephrotic syndrome, associated with glomerulonephritis, amyloid disease, renal vein thrombosis, diabetes and hypertension. The microscope can be used for ordinary purposes by making the analyzer and polarizer parallel.—John T. Myers.

LOTZE, J. C., and YIENGST, M. J. An apparatus for measuring microscopic objects. *Science*, 93, 45-6. 1941.

A device is described for obviating the shortcomings of micrometers and other scales when measuring objects less than 1μ in diameter. Essentially, the apparatus consists of a microscope, the prism of a camera lucida, and a screen. The latter covers a lighted box, and is pierced with calibrated holes and slits that represent, as viewed thru the microscope, established lengths with which the size of microscopical objects can be compared and gaged.—J. A. de Tomasi.

MARTON, L. The Electron Microscope. *J. Bact.*, 41, 397-413. 1941.

A description with diagrams of the instrument, a chart of its resolving power, photographs, and bibliography are included.—Virgene W. Kavanagh.

POHL, H. A. Convenient heat filter for tissue illuminator. *Science*, 92, 612. 1940.

The quartz rod illuminator of Knisely used in the observation of tissues *in situ* transmits a considerable amount of heat from the illuminating bulb. The inconvenience of using a continuous stream of Ringer's solution as a cooling agent is eliminated by inserting a double thickness of inexpensive "heat filter" glass (Cenco 87305) at the break in the rod light conductor. The heat transmitted is cut to at least $1/7$ its previous value, while the light intensity is reduced by only a small fraction.—J. A. de Tomasi.

RHEA, H. E. The new electron microscope. *Science*, 93, 357-8. 1941.

The first electron microscope represented a major step toward fundamental developments in the field of microscopy; with it, particles of 50 \AA in diameter could be clearly resolved. But that first apparatus was bulky and cumbersome, it required an installation free from vibrations and magnetic fields, and necessitated the services of an electronic engineer to operate it. The latest model is designed to fit into a small room and to be simple in operation. The microscope, including its power supply, is contained in one rack 7 ft. tall and weighs about 500 lb. A normal 110-volt line socket supplies the current.—J. A. de Tomasi.

SMITH, K. U. A dry-ice freezing unit for cutting frozen sections. *Science*, 92, 364. 1940.

This unit is designed to obviate the shortcomings of commercially available attachments, and to meet such needs as sectioning of large specimens, and prolonged and uniform freezing. It consists briefly of a cylindrical cast-aluminum container of 8.4 cm. in diameter with a slightly raised and grooved center table 5 cm. in diameter. A hexagonal bakelite support is fitted underneath for clamping into the holder of the microtome. The tissue rests on the table, and powdered dry ice is packed around and under it within the container. This unit may be obtained from M. Hanford, Physics Department, University of Rochester, Rochester, N. Y.—*J. A. de Tomasi*.

MICROTECHNIC IN GENERAL

BUCHOLTZ, IRENE. Cellophane coverslips and a method for mounting. *Science*, 92, 436. 1940.

Plain cellophane (No. 900) appears to be satisfactory as a substitute for cover glasses which are becoming increasingly scarce and expensive. This material has a refractive index of 1.53, comes in rolls 15/16 in. wide, and is 0.022 mm. thick. In favor of using it in microtechnic are its thinness, low cost, rapid drying, and the fact that it does not curl. It also allows taking photomicrographs, and the use of oil immersion except in the case of very high magnifications. One difficulty, however, lies with the problem of storing the cellophane. It tends to absorb moisture and the edges of the roll then wrinkle. Storage in a dessicator for long periods causes warping and cracking. Moisture-proof cellophane is less desirable than plain cellophane.

For mounting slides with glass or cellophane cover slips, proceed as follows: turn the slides face down on paper towels, pipet xylene between them, and cover with another towel. In 15–30 min. the preparations are ready for use.—*J. A. de Tomasi*.

GROAT, R. A. Evaluation of isobutyl methacrylate polymer as a mounting medium. *Science*, 92, 268. 1940.

This is a discussion of the value of isobutyl methacrylate polymer as a mounting medium principally from the point of view of relative refractive indices. These values are as follows: unstained striated muscle, 1.537 ± 0.003 ; Canada balsam, 1.535; gum damar, 1.542; Clarite, 1.544; Clarite X, 1.567; isobutyl methacrylate polymer, 1.477. Since the refractive index of stained tissue is still higher than that of unstained tissue, the low value for the polymer appears still less compatible with the prerequisites of the dispersion curve obtained by plotting refractive index against wave length. An ideal mounting medium cannot be realized because different tissue elements do not have the same dispersion curves; thus for practical purposes a medium should be used that has a refractive index for the D line of the spectrum as close as possible to that of most of the stained tissue elements. The use of the polymer for mounts with or without cover glass is not recommended for other reasons: it does not adhere to glass as well as other resins; certain stains fade rapidly in it; and it softens and decomposes at relatively low temperatures.—*J. A. de Tomasi*.

GROAT, R. A. New paraffin resin infiltrating and imbedding media for micro-technique. *Science*, 93, 311–12. 1941.

A series of mixtures of paraffin (m.p. 56°–58° C.) is here reported to mix well with water-clear, LX-291, hydrocarbon resin produced by the Neville Co., Pittsburgh, Pa. Chief advantage is that by changing proportions in the mixtures, the resulting media differ appreciably in hardness at a given temperature, but not essentially in melting range. Hardness increases as the resin content rises. Concentrations suitable for practical application, containing 5, 10, 20, and 30% resin, show a melting range of several degrees, with a minimum around 55° C. An average oven temperature of 59° C. is satisfactory for all. The mixtures are prepared as follows: Blend the weighed portions of paraffin and resin at 170° C., and filter. Infiltrate in pure paraffin, or 5–10% mixtures; embed in a harder medium. For comparison, a 10% medium yields satisfactory ribbons in the range between 4 and 12 μ ; a 50% medium permits only 1 μ ribbons.—*J. A. de Tomasi*.

McDOWELL, A. M., and VASSOS, G. A., Jr. Comparison of starch paste and albumin mixture as agents for the routine mounting of paraffin sections. *Arch. Path.*, 29, 432. 1940.

Albumin-glycerin mixture is made by taking equal parts of egg white and glycerin, filtering and adding a small crystal of thymol as preservative. Starch paste is made by adding 10 cc. of cold water to 1 g. of powdered starch and mixing thoroly. The mixture is then poured in 20 cc. of boiling water, 2 drops of dilute HCl are added and the suspension is boiled with constant stirring for 5 min. A small crystal of thymol is added when the paste is cool. After mounting the sections, the slides are submitted to various forms of drying; at 56°–58° C. for 48 hr. or at 38°–50° C. for 5 days is adequate for subsequent staining with silver but unnecessary for other technics; drying on a hot plate for 8 min. and in drying oven for 1 hr. at 56°–58° C. is adequate for Ziehl-Neelsen with albumin but not with starch. (From *J. Royal Micro. Soc.*, 60, 262, 1940.)

RANDALL, A., and MENZIES, A. W. C. Histological sectioning of hard tissues by a new technique. *Science*, 93, 189–90. 1941.

The use of fluid methacrylates is suggested as a possible means of obtaining sections of hard and brittle materials like bone tissue. The resin is polymerized to a solid embedding medium *in situ*, and sections are ground from the specimen by petrographic technic. The procedure is as follows: Embed dry materials directly; or dehydrate (in alcohol, acetone, or dioxan), and clear in xylene. Impregnate in a test tube (sometimes at reduced pressure) in monomeric methyl methacrylate for 12 hr., changing it three times with 0.05% benzoyl peroxide added as a catalyst for polymerization. Heat a portion of the catalyzed monomer for 24 hr. at 40° C. Free the hardened contents of the test tube by breaking the glass, and proceed with the grinding. If serial sections are desired, cut first thin wafers and affix to "plexiglass" slides with the partial polymer; complete the polymerization for 6 hr. at 40° C. Sections obtained by this method are reported to stain well with safranin and methylene blue.—*J. A. de Tomasi*.

SUNTZEFF, V., and SMITH, IRENE. The use of plastic as a substitute for coverglasses. *Science*, 93, 158–9. 1941.

These remarks are an extension to a note in *Science*, 92, 17–8, 1940. It is stated that hematoxylin eosin stains mounted in Canada balsam will fade within 4–5 months when mounted under plastic covers.—*J. A. de Tomasi*.

DYES AND THEIR BIOLOGICAL USES

VARCO, RICHARD L., and VISSCHER, M. B. Further studies in the elimination of certain dyes by gastric mucosa. *Proc. Soc. Exp. Biol. & Med.*, 46, 295–8. 1941.

The following list of dyes reported to be secreted in gastric juice has been re-investigated: Congo red, orange G, mercurochrome, erythrosin, phloxine, rose bengal, lithium carmine, trypan red, eosin, and chrysoidin. None of them appeared in histamine-excited gastric juice from Pavlov or Heidenhain pouches in dogs. This work gives support to the earlier generalization that only dyes in which the chromogen may be in the cation appear in highly acid gastric secretions.—*M. S. Marshall*.

ANIMAL MICROTECHNIC

BAXTER, J. S. The application of trichrome staining methods to embryological technique. *J. Anat.*, 75, 187–40. 1940.

This paper is a summary of seven years experience with trichrome staining methods in the preparation of several mammalian embryonic forms for teaching and research. A method for the preliminary treatment of embryos is given. Detailed procedures for two trichrome staining methods are outlined, together with the author's notes and comments. The author recommends for young embryos the use of an iron-hematoxylin nuclear stain followed by a simple cytoplasmic counterstain such as orange G in 1% solution. The embryos used in the above study were those in which epithelial and connective tissue differentiation had commenced.—*F. M. Clark*.

COULSTON, FREDERICK. The use of diaphane for mounting Giemsa type preparations. *Amer. J. Clin. Path.*, 26, 869-73. 1941.

Smears and dehydrated sections often fade when mounted in balsam due to formation of acid by oxidation. Diaphane does not become acid. It is unconditionally recommended instead of balsam or cedar oil for mounting preparations with any Romanowsky type stain.—*John T. Myers*.

GORDON, H. K., and CHAMBERS, R. The particle size of acid dyes and their diffusibility into living cells. *J. Cellular and Comp. Phys.*, 17, 97. 1941.

A study was made of the relation of the particle size of a number of water-soluble acid dyes to their penetration into three types of living cells. The tissues used were muscle fibers of the gastrocnemius, ciliated epithelial cells of the oral mucosa and sheets of bladder epithelium of the frog *Rana fusca*. Dyes with a particle radius of 5.5 ± 0.4 to 6.4 ± 0.5 Å (when diffusing in Ringer's solution at pH 7.4 to pH 6.8) or less penetrated the ciliated epithelial cells; the upper limit of size for the muscle fibers or the bladder epithelium was 6.5 ± 0.4 to 7.3 ± 0.9 Å. No definite relation was observed between lipid solubility and the penetrability of the dyes. No difference in penetration of the dyes was observed for cells under a nitrogen or an aerobic atmosphere.—*L. Farber*.

KATZ, ALBERT. A contrast staining method for hemosiderin pigment in heart-failure cells. *J. Lab. & Clin. Med.*, 26, 700. 1941.

There has been no satisfactory method for differentiating heart failure cells, in sputum or urine, from the surrounding leucocytes. This can be done by the following technic:

Make smear of sputum as usual, air-dry, fix with heat, apply 10% K_4FeCN_6 and an equal quantity of 10% HCl. Let stand 15 min., wash with water and dry. Apply Wright's stain and add buffered water for 5 min. Wash with water, dry and examine under oil. The hemosiderin takes a bright green stain.—*John T. Myers*.

LENDRUM, A. C. The preparation of tissues for paraffin embedding. *J. Path. & Bact.*, 52, 138-42. 1941.

The author finds Stiles' butanol dehydration series (Stain Techn., 9, 97, 1934) the most satisfactory among a wide variety of methods for preparing easily sectioned paraffin blocks. Tissue can be left for days in the dilutions without harm, and a prolonged stay afterwards in the paraffin oven does no harm. Better results with dense tough tissues can be obtained by following butanol dehydration with pyroxylin infiltration as follows: equal parts of butanol and methyl benzoate, one day; methyl benzoate, one day; then 7-14 days in methyl benzoate, containing 2% pyroxylin and 1.5% tricesyl phosphate; two changes of chloroform, over a period of 5 hr., to precipitate the pyroxylin; infiltration with paraffin for 6-48 hr. or longer.

Low viscosity nitrocellulose can be used if combined with sufficient plasticiser, by the following technic: Pass tissues from butanol, thru two graded mixtures, to pure cellosolve, then to a 15% solution of collodion cotton in cellosolve containing 14% tricesyl phosphate, and leave for 7-30 days. Then treat with chloroform and imbed in paraffin as usual. Without plasticiser the collodion cotton is much too hard.—*S. H. Hulme*.

MULLEN, JOHN P., and McCARTER, JOHN C. A mordant preparing formaldehyde-fixed neuraxis tissue for phosphotungstic acid hematoxylin staining. *Amer. J. Path.*, 17, 289-91. 1941.

The authors propose a method of mordanting formalin-fixed tissue, even after storage in formalin for several years, which yields results equivalent to those obtained on tissue fixed in Zenker's fluid. The procedure is as follows: Wash the fixed tissue thoroly and prepare it for paraffin sections in the usual manner. After cutting and affixing the sections to the slide, remove paraffin and pass the slides thru graded alcohol to water. Mordant for 2-48 hr. in a 5% solution of $CrCl_3 \cdot H_2O$ (green crystals; obtainable from the General Chemical Co., New York, N. Y.) in distilled water to which 5% glacial acetic acid is added. (This mordant is stable and will keep for weeks, altho the color changes.) Rinse, after mordanting, in distilled water, treat with 0.25% aqueous $KMnO_4$ for 10-15 min., rinse again,

and bleach in 5% aqueous oxalic acid until the sections have lost the brown color; rinse and stain with Mallory's phosphotungstic acid hematoxylin (either the newer or older formula) for 6-12 hr. Decolorize in 95% alcohol, 2 or 3 changes; dehydrate; clear and mount. This method is suited for routine use on brain and spinal cord.—*H. A. Davenport.*

OTTO, REDGINAL HEWITT, OTTO, C. F., and STRAHAN, DOROTHY E.
A simplified zinc sulfate levitation method of fecal examination for protozoan cysts and hook worm eggs. *Amer. J. Hyg.*, 33, 32-7. 1941.

The technic is that of the Willis brine levitation method (*Med. J. Austr.*, 1921, 375-6) except that 1.18 sp. gr. $ZnSO_4$ is substituted for saturated $NaCl$ solution. A portion of feces is placed in a 1 oz. metal container 3.8 cm. in diameter and 2.8 cm. deep. It is completely filled with the $ZnSO_4$ solution, covered with a glass slide and let stand 1 hr. The slide is removed, inverted and examined. The method cannot be used for the vegetative stage of amoebae.—*John T. Myers.*

SALTER, W. J. A haemocytometer method for reticulocyte and platelet counts. *J. Path. & Bact.*, 52, 148-50. 1941.

Reticulocytes and platelets can be counted simultaneously and rapidly by the following accurate method: Prepare the diluting fluid by mixing two parts of an aqueous solution of brilliant cresyl blue (1:300) with three parts of a 1:1,400 aqueous solution of KCN, the final solution being centrifuged at high speed for 5 min. (The two solutions must be kept separate and mixed immediately before use.) Take blood to the mark 0.5 in a white cell pipette, dilute to mark 11 with the diluting fluid, and shake well. Place a drop in the counting chamber and count after 5-10 min.

Platelets appear as ovoid blue or lilac-colored bodies. Reticulocytes are recognized as usual. A leucocyte count can be made at the same time.—*S. H. Hutner.*

SMITH, KATHERINE EDSALL. The measurements of red blood cell diameter by the diffractometer. *J. Lab. & Clin. Med.*, 26, 696-699. 1941.

The Spencer Lens Co. manufactures a diffractometer which may be attached to any monocular microscope. A small black cylinder with a pinhole in the bottom is attached in place of the objective. A second cylinder with a pinhole in the top and an adjustable device on the side for measuring the halo replaces the ocular. A stained or unstained slide may be inserted into a slot in the top cylinder with the smear down. The smear should be only one cell thick, and should be observed with maximum light. A central spot of light should appear, surrounded by a yellow halo which shades into a red periphery. The two parallel black bands should be adjusted on the outside so that they just touch the outer rim of the red halo. The reading is taken from the sliding scale and the mean cell diameter determined from a conversion table. This method is useful in determining macro- or microcytosis.—*John T. Myers.*

TARAO, S. Microchemical studies on the Golgi apparatus using protease Nile blue sulfate technique. II. Golgi apparatus of pancreatic acinar cells in the mouse in fixed and living conditions. *Cytologia*, 11, 261-81. 1940.

Anilin dyes are not ordinarily considered useful for staining the Golgi apparatus, but Nile blue sulfate was used to demonstrate it in a series of papers by Tarao of which this is the third. The protease Nile blue sulfate technic is applied as follows: Fix small pieces of the pancreas of a mouse not fed for 10 hr. (anaesthetized with chloroform) in 20% neutral formalin (8% formaldehyde) for 24 hours or more. Make 20 μ sections on a freezing microtome. Digest for about 16 hr. at 37° C. in a solution of 0.3 g. trypsin and 0.8 g. anhydrous Na_2CO_3 in 100 cc. distilled water, to remove much of the protein from the acinar cells and the masked proteins of the Golgi apparatus. Wash thoroly in distilled water and stain over night in 0.01% aqueous Nile blue sulfate (Grübler's). Differentiate in 1% aqueous acetic acid until the sections are faded to a cobalt blue. Wash thoroly in distilled water. Mount in a saturated aqueous solution of dextrose and seal with vaseline-paraffin in equal parts. The Golgi substance stains pink-blue; other fatty substances stain red or blue according to their degree of saturation; the cytoplasm is unstained. The Golgi apparatus is the same shape and size as it is after metallic impregnations. Pepsin solutions may be used to digest the

hepatic cells of mouse and newt but do not digest the pancreatic cells. Other fat stains may be substituted for the Nile blue sulfate; Smith-Dietrich's hematoxylin, Ciaccio's Sudan III, and Fischler's copper-acetate-hematoxylin give rather good results.

Intra-vital staining of the Golgi apparatus is secured by injecting 0.6-0.7 cc. of 0.2% Nile blue sulfate in normal saline intraperitoneally in the mouse. (A slightly larger dose would be fatal.) In about 90 min. the Golgi apparatus develops a faint blue tinge which lasts not longer than 20 min.; the bits of pancreas must be removed, mounted, flattened, and examined within this period. Polarized light is useful for viewing these slide preparations of bits of the pancreas. Thru a Zettnow's green filter, which is made up of CuSO₄ and CrO₃ in distilled water, the blue color of the Nile blue sulfate appears black. Other small granules (Ries' Lyrochondria) are stained a dark blue in the same preparations.—*Virgene W. Kavanagh*.

WETMORE, PSYCHE W. A direct method of determining the erythrocyte, leucocyte and thrombocyte count of fowl blood. *Science*, 92, 386. 1940.

This technic is a modification of the Blain method, and affords rapid and reliable enumeration of the cellular elements of avian blood in the counting chamber. The procedure is as follows: Prepare a staining stock solution by mixing 1 cc. 1% aqueous brilliant cresyl blue with 0.25 cc. saturated aqueous pyronin (6.7%); dilute 0.2 cc. of this in 25 cc. normal saline, and filter thru neutral paper. Draw blood from the wing vein up to the 1 mark in the red cell counting pipette. Fill it half way with the dye-saline solution, rotate 5 sec., and fill to the 101 mark with 12% formalin in Locke's solution, as a preservative. Shake for 30 sec.; for better differentiation, allow it to stand 15 min. or longer. Use thin covers, and examine under a 4 mm. objective. The dye solution keeps for about a week, while the stock solution is less stable; the formalin solution must be made fresh each day.—*J. A. de Tomasi*.

WU CHOA-FA. Laboratory technique notes. III. The use of alum-hematoxylin for staining in bulk. *Peking Nat. History Bull.*, 15, 195-7. 1940.

Most methods of staining large blocks of tissue have used carmine or have required an extensive treatment of the slides after sectioning. The following method apparently gives well-stained sections without any treatment which might wash them off the slides: Place blocks of animal tissue, that have been fixed in Bouin's fluid, washed, and decolorized as usual, in about 20 volumes of hematoxylin solution (8 cc. of Ehrlich's stock solution of hematoxylin, 30 cc. of 50% alcohol, and 2 cc. of glacial acetic acid) for 2-5 days, 2 days for a piece 3 mm. on an edge, 5 days for a centimeter cube; stir occasionally; rinse the block in water; to remove the excess stain, soak it in 3 or more changes of weak acetic alcohol (5 cc. of 10% acetic acid in 100 cc. of 30% alcohol) until the last change remains almost colorless for $\frac{1}{2}$ hr.; wash in running tap water over night; and dehydrate in a series of alcohols to 95%. If a counterstain is desired, place the block in a 0.2% solution of alcohol-soluble eosin in 95% alcohol, then in a 0.2% solution in absolute alcohol, 12 to 24 hr. in each. After the blocks have been stained, they may be cleared and embedded as usual altho the following method is suggested: Pour a layer of chloroform twice as deep as the thickness of the block into a vessel; on top of this pour a similar layer of methyl salicylate; and on this pour a layer of absolute alcohol; put the block into the alcohol layer and let it remain in the vessel until it has sunk into the chloroform; put it into paraffin-chloroform mixtures, then into paraffin as usual for embedding. The methyl salicylate layer may be omitted for small embryos and delicate tissues. After the blocks are embedded, section and mount as usual; then remove the paraffin with xylol, run the slides to absolute alcohol for 1 min. or more to differentiate the eosin; run them back thru xylol and mount in balsam.—*Virgene W. Kavanagh*.

ZAHL, P. A., and COOPER, F. S. Localization of lithium in tumor tissue as a basis for slow neutron therapy. *Science*, 93, 64-5. 1941.

It has been repeatedly observed that certain acid dyes, when introduced into the blood stream, accumulate preferably in-cancer tumor tissue. Since most of these dyes are sodium salts of the azo-sulfonic complex, the effect of substituting lithium for sodium in the dye-salt molecule is investigated. The reason for doing

this is that slow neutrons projected thru body tissue cause no damage when they encounter a zone perfused with such elements as boron or lithium. In this case nuclear capture reactions release protons of high energy and the result is local destruction of tissue, suggesting application of a neutron-boron or neutron-lithium technic for localized treatment of tumors. Lithium salts of pontamine sky blue 6B, trypan blue, and carminic acid (duPont and National Aniline Co.) were prepared and injected intravenously in mice with spontaneous and implanted mammary tumors. Spectroscopical analysis of tissues removed later proves that a localization factor of approximately 2 is attained, with a maximum concentration of 0.01–0.03% lithium in the tumor mass. The addition of lithium-ions results in a net increase in absorbed energy corresponding to a maximum gain of about 48% in the radiation dosage of the tumor over that of other tissues in the same mouse.—*J. A. de Tomasi.*

MICROÖRGANISMS

BESTA, B. L'uso del microscopio a fluorescenza nella ricerca del bacillo di Koch. *Ann. d. Ist. Carlo Forlanini*, 3, 904. 1939.

The method of Keller was used, with a 2:1000 solution of auramin. In 200 examinations of material already known to be positive by the Ziehl-Neelsen method, the fluorescence method gave the same positive results, but required less time for the examination of the slides because a larger microscopical field could be covered by the use of small power objectives, i. e. 10 \times or 20 \times . In 241 examinations of previously negative material, 7% were positive by the Ziehl-Neelsen method, 18.7% by the fluorescence method, and 84% by culture on Petagnani's medium or by guinea pig inoculation. This method is time-saving, more accurate and economical, because in less than 50% of cases biological proof is needed. Occasional doubtful results may be had with the fluorescence method, but this is also true of the usual staining methods.—*S. Lojacono.* (From *Abst. sect. of Amer. Rev. Tuberc.*, 43, No. 4, 1941.)

DIDION, H. Über den fluoreszenzmikroskopischen Nachweis von Tuberkel-bakterien. *Klin. Woch.*, 18, 1915. 1939.

Fluorescence microscopy has been employed as an alternative method of identifying tubercle bacilli on slides. The preparation is stained with auramin, a yellow anilin dye; when viewed under the microscope, the organisms are colored a yellowish green. In order to determine the accuracy of this method, 702 specimens, including sputum, pus, urine, feces and other body fluids of suspected tuberculous nature, were examined for tubercle bacilli by four methods: animal inoculation, culture (according to the method of Löwenstein, Lockemann and Hohn), Ziehl-Neelsen stain, and auramin stain according to the method of Hagemann. Altogether there were 157 positive specimens: 129 by the Ziehl-Neelsen method; 137 by the auramin method; 12 were discovered by cultural methods; and 8 only by animal inoculation. Of the 157 positive specimens, the following figures show the percentage accuracy of the various methods: animal inoculation, 100; culture, 99; auramin, 87.2; Ziehl-Neelsen, 82.2. Of the staining methods the auramin fluorescence method is not only somewhat more accurate but is also definitely time-saving, particularly in searching for tubercle bacilli in tissue preparations.—*H. R. Nayer.* (From *Abst. sect. of Amer. Rev. Tuberc.*, 43, No. 4, 1941.)

KNAYSI, G. Observations on the cell division of some yeasts and bacteria. *J. Bact.*, 41, 141–53. 1941.

To stain the cell walls and slime-layers, which are rarely seen in ordinary preparations, the following procedure is recommended: Smear a young culture (only a few hours old) and fix it over the flame; mordant it for 10 min. with a mixture of 70 ml. saturated aqueous $\text{AlK}(\text{SO}_4)_2$, and 30 ml. of 20% aqueous tannic acid; stain with Ziehl-Neelsen's carbol fuchsin under a cover glass. The cytoplasm is dark red; the cytoplasmic membrane, still darker; the cell wall, blue; and the slime-layer, bright red. If the preparation is sealed with vaspar, a mixture of 50% paraffin and 50% vaseline, it will keep for a considerable time. A fresh mordant mixture should be prepared every two weeks.—*Virgene W. Kavanagh.*

LIPP, HANS. Eraparnisse bei der Gonokokken- und Spirochätenfärbung. *Münch. Med. Woch.*, 87, 888. 1940.

Staining procedures for gonococci and spirochaetes are given—the former an adaptation of the Thimsche technic.

The procedure for gonococci is as follows: Prepare a thin smear of the secretion and fix by passing it three times thru a flame; stain 30 sec. in a solution made up of equal parts of 1% aqueous methylene blue and 1% aqueous gentian violet solutions; wash and dry. The gonococci stain dark violet, the nuclei of leucocytes appear dark blue, the cytoplasm of leucocytes stains pale reddish-violet and the nuclei of epithelial cells deep blue.

Four staining procedures are described for spirochaetes, particularly for *Treponema pallidum*. These are as follows:

1. Fix smear in methyl alcohol; treat with a few drops of 1% KOH, followed immediately by a few drops of a dilute fuchsin solution (a 1:20 dilution of a saturated fuchsin solution in 95% alcohol) which becomes cloudy after 1 min. and is decolorized after 3 min.; wash in water and dry. (The red cells of *Treponema pallidum* are easily distinguished from the pale red background.)

2. Fix smear by drying in air, stain 3 min. in a 5% aqueous KMnO₄ solution; wash with water; counterstain 2 min. with a 1:10 carbol fuchsin solution. (The *Treponema refringens* and mouth spirochaetes stain a deep red brown, *T. pallidum* stains somewhat lighter but still distinguishable.)

3. Prepare a hanging drop of the serum obtained by the irritation of an infected area; add 1 or 2 loopfuls of Loeffler's methylene blue; mix and add 1 loopful of N/10 NaOH. (*Treponema pallidum* appears sky blue and is to be found particularly at the edge of the drop.)

4. Air-dry the smear; treat with 3% aqueous Victoria blue IV R (Grübeler) 3 min.; wash carefully and dry in the air. The characteristic morphology of *Treponema pallidum* is clearly distinguishable from that of any other spirochaetes which may be present. A more intensely staining solution may be prepared as follows: Victoria blue IV R, 3 parts, pyronin 0.9 parts, methyl green 0.1 part, absolute alcohol 9 parts, glycerol 10 parts and distilled water to 100 parts. This solution stains *Treponema pallidum* a deep blue-black color in 3 min.—L. Farber.

MALLMANN, W. L., and DARBY, C. W. Uses of lauryl sulfate tryptose broth for the detection of coliform organisms. *Amer. J. Pub. Health*, 31, 127-34. 1941.

The addition of sodium lauryl sulfate, a surface tension depressant, to lactose broth gives a medium selective for the coliform group. The base medium consists of 2% Bacto tryptose; 0.5% NaCl, 0.4% K₂HPO₄, 0.15% KH₂PO₄, 0.5% lactose. To this is added sodium lauryl sulfate, as Duponol W. A. Paste or Nacconol N. R. F. S., to give a concentration of 1 part in 10,000. A comparison was made by testing three media in parallel, namely, standard lactose broth, lactose tryptose broth, and lauryl sulfate tryptose lactose broth. Most of the studies were on raw waters. Confirmation was carried out in either brilliant green bile broth or E. M. B. agar or in both. The results showed that, with unpolluted tap waters, standard lactose broth gave many more positive presumptive tests which failed to confirm than did lauryl sulfate broth or tryptose broth. With polluted raw waters, there was little difference between lactose broth and lauryl sulfate broth as regards number of positive gas tubes and tubes confirmed, altho the latter allowed the growth of more gas producers than did the former. The confirmatory media often acted as suppressing agents to coliform organisms, giving a lower coliform index than would be the case with more suitable confirmatory media. Tryptose broth was a better confirmatory medium than brilliant green broth. When gas was produced in lauryl sulfate broth, confirmation was always obtained.—M. W. Jennison.

MAYFIELD, CATHERINE R., and GOBER, MAUD. Comparative efficiency of plating media for the isolation of *Shigella dysenteriae*. *Amer. J. Pub. Health*, 31, 363-8. 1941.

This paper reports the comparative efficiency of plain Endo, lithium chloride Endo, desoxycholate-citrate, bismuth sulfite, and *Shigella Salmonella* culture media for the isolation of *Shigella dysenteriae* from routine feces specimens re-

ceived at the laboratories of the Mississippi State Board of Health. The Shigella Salmonella medium is a recent product of the Difco Laboratories. Specimens were inoculated on the plating medium used for isolation the day they were received, and repeated platings made on the second and third days. After 18 hours incubation suspicious colonies were fished to triple sugar tubes, and colonies positive on this medium were inoculated into various carbohydrate media. Motility tests, as well as indole determinations and macroscopic slide agglutination tests, were made. Of 435 positive cultures isolated on one or more of the media, there were 95% positive on desoxycholate-citrate, 48% on plain Endo, 20% on lithium chloride Endo, and 2.3% on bismuth sulfite; of these, 175 were positive only on desoxycholate-citrate, compared with 14 from plain Endo, 4 from lithium chloride Endo, and 2 from bismuth sulfite agars.—*M. W. Jennison.*

MIYAHARA, H. The quick staining method of the malaria parasite by means of Giemsa's stain. *Acta Jap. Med. trop.*, 1, 49-55.

The dried unfixed film is hemolysed in a beaker of water, the film side of the slide being uppermost. The solvent of the stain, 0.003-0.005% K_2CO_3 or Na_2CO_3 solution, is heated to about 60° C. One drop of the Giemsa stain is added to each cc. of the heated solvent; 3 cc. of the warm solution is poured on the film and left for 5-10 min.; the film is then washed and dried. Schüffner's dots are well stained but appear finer than after alcohol fixation. The parasitized cells and the parasites are well stained. No stain granules are deposited on the film. (*From J. Roy. Micr. Soc.*, 60, 262, 1940.)

MONK, C. R. Marine harpacticoid Copepods from California. *Trans. Amer. Micr. Soc.*, 60, 75-99. 1941.

The harpacticoids, usually killed by collecting them in fresh water or in 2% formalin, were stained with fast green (no other information given) in 70% alcohol for 1 hr. before dissection. The addition of a few drops of Bouin's fluid to the stain aided in distinguishing fine setae. They were dissected in glycerin.

Permanent mounts of the appendages were made in a syrup-pectin mixture (white Karo syrup, 5 cc.; Certo, 5 cc.; water, 3 to 5 cc.), dried over heat, covered with euparal and bits of cover glass.—*Virgene W. Kavanagh.*

RICHARDS, O. W. The staining of acid fast tubercle bacteria. *Science*, 93, 190. 1941.

Of the many compounds isolated from tubercle bacteria, mycolic acid alone was found to be acid-fast; but the degree of fastness varies greatly according to the dye. The carbol fuchsin of the Ziehl-Neelsen method stains it with difficulty, and is easily washed out in acid alcohol; carbol auramin, on the other hand, stains the acid an intense fast yellow. While mycolic acid is weakly fluorescent to ultra-violet irradiation, auramin causes it to show bright yellow fluorescence. The suggestion is made that mycolic acid may be the source of acid-fastness, and may be responsible for higher counts in the fluorescence technic.—*J. A. de Tomasi.*

RICHEY, DALE. Relative value of 2% and 5% brilliant green bile confirmatory media. *J. Amer. Waterworks Assoc.*, 33, 649-58. 1941.

It was found that this medium was specific for the coliform group of organisms, with either 2% or 5% brilliant green. The latter was superior to the former in (1) the number and proportion of coliform organisms isolated, (2) giving the smallest total cumulative error, and (3) inhibiting spore-forming organisms.—*Merritt N. Pope.*

SCHLEIFF, P. Zur Pilzfärbung, insbesondere der Erreger der Epidermophytien der Hände und Füsse. *Münch. Med. Woch.*, 87, 785. 1940.

A method is described for the preparation and staining of samples for the detection of epidermophytic infections.

Preparation of material. Scraping from around the margin of non-purulent areas of infected skin or the tops of vesicles are transferred to a slide. They are treated 3 to 10 min. (depending on the size and thickness of the specimens) with Carnoy's solution (glacial acetic acid 10 ml., chloroform 80 ml., absolute alcohol to make 100 ml.). This treatment serves to swell the horny substance of the skin,

to remove any fatty material and to dehydrate the sample. The Carnoy solution is poured off carefully in order to carry away any floating debris and leave the fungus adhering to the slide. Dry carefully over a flame.

Staining Procedure. Treat with a 0.5% aqueous Azure I solution 2-3 min.; wash carefully with distilled water, adding the water drop by drop to avoid washing away the sample. Dry over a flame and mount in Canada balsam.

The fungal filaments stain dark blue and are easily distinguished from the light blue background.—*L. Farber.*

SCHNEIDER, P. P. Fluoreszenzmikroskopie und Tuberkelbazillennachweis im Tuberkulosenkrankenhaus. *Zts. Tuberk.*, 84, 319. 1940.

The advantages of the fluorescent microscope in demonstration of tubercle bacilli have been previously reported. There is considerable saving of time and less fatigue. In a series of 1100 examinations there were 22.7% more positive results with the fluorescent microscopy than with the Ziehl-Neelsen method. Negative in relation to positive results were: for sputum, 3:4 with Ziehl-Neelsen, and 3:5.5 with the fluorescent microscopy; for antiformin concentrate, 3:0.9 and 3:1.4; for gastric juice, 9:1 and 8:1. The use of the method in the tuberculosis hospital is highly recommended, but it cannot replace culture and animal expts.—*G. C. Leiner.* (From *Abst. sect. of Amer. Rev. Tuberc.*, 43, No. 4, 1941.)

STANLEY, I. N. Development of the basidium of *Eocronartium muscicola*. *Trans. Amer. Micr. Soc.*, 59, 407-18. 1940.

Dried basidia have been stained well with phloxine by the following technic: Soak sporophores for a few hours in water; tease out minute particles of the hymenium on a slide; add 3% KOH; add 1% phloxine (no other specifications); cover; remove excess phloxine by pulling KOH under the cover glass with filter paper. By this method, basidia are bright rose; sterile hyphae, light pink. Lactic acid proves useful in swelling the dried material.—*Virgene W. Kavanagh.*

STARR, M. P. Spirit blue agar: a medium for the detection of lipolytic micro-organisms. *Science*, 93, 393-4. 1941.

This is a medium claimed to be highly differential and at the same time to be capable of allowing growth of the more delicate microorganisms as well. The preparation of the medium is as follows: Dissolve 30 g. agar, 10 g. tryptone, and 5 g. yeast extract in approximately 900 ml. distilled water by autoclaving; to this add 25 ml. of a 20% cottonseed oil emulsion (10 g. powdered gum arabic ground thoroly in 100 ml. Wesson oil and 400 ml. warm distilled water), and 50 ml. of 0.3% freshly filtered alcoholic spirit blue (National Aniline); bring up to 1000 ml. with distilled water, and autoclave 15 min. at 15 lb. (121° C.). The medium must be stored in a refrigerator till used. Plates made from it appear pale lavender; lipolysis is indicated by a permanent deep blue beneath and around the colony.—*J. A. de Tomasi.*

TYAGARAJA, S. A simple modification of Wilson and Blair's medium for the isolation of typhoid and paratyphoid bacilli. *J. Hygiene*, 40, 414-22. 1940.

To heart infusion agar at 60° C. is added, per liter, 0.1 cc. of 1% brilliant green, 4 cc. of 12% fresh bismuth ammonium citrate and 4 cc. of 40% Na₂SO₄, shaking after each ingredient is added. Glucose, phosphate and FeSO₄ are eliminated. Good keeping qualities, inhibition of *Aerobacter aerogenes*, and differentiation are claimed.—*M. S. Marshall.*

WENRICH, D. H. The morphology of some protozoan parasites in relation to microtechnique. *J. Parasitology*, 27, 1-28. 1941.

The author emphasizes the importance of properly prepared slides as an aid in the diagnosis of intestinal protozoa, and discusses the value of modifications of the usual technics.

Fixation for 1 min. gives results as good as or better than longer times for the fixing agents commonly employed for intestinal protozoa. Schaudinn's fluid and other fixatives can be greatly diluted without destroying their effectiveness as fixing agents.

Different races of the same species may have different appearances with the same technic. Different individuals on the same slide may show different reactions to the same technic. On the other hand, different fixing solutions and different stains may produce decidedly different appearances for the same species, as, for example, *Trichomonas muris*. Different species show both chemical and morphological differences in the composition of their nuclei, a point particularly well shown when the Feulgen reaction is applied to the intestinal amoebae of man and the results compared with those obtained with the more common stains.—*Elbert C. Cole.*

HISTOCHEMISTRY

SEKI, M., and KOHASHI, Y. Bestimmung des isoelektrischen Punktes der Gewebeelemente auf färberischem Wege. I. Ergebnisse der pH-Bestimmung in Anwendung verschiedener Puffergemische. *Folia Anat. Jap.*, 19, 47-52. 1940.

The authors have investigated the variable results obtained by others with the Fischinger's colorimetric method. They arrive at the conclusion that the composition and concentration of the buffer mixtures play an important role in the colorimetric isoelectric point determination (denoted by the authors as pH) of tissue elements.

Clark's phthalate buffers, mixed with an equal amount of dye solution, proved the best, because their negativating anion action upon the tissue element charge was the lowest within the range pH 2.5-6.0. McIlvaine's Na₂HPO₄-citric acid mixture (diluted 5 times) served almost equally well.

Pieces of rabbit kidney, duodenum, and liver were used as test objects because of their loose texture and lipoid scarcity. Blocks were fixed 24 hr. in 95% alcohol or 10% formalin, passed thru 80%, 95%, and absolute alcohol into Dekalin-methylbenzoate-paraffin. Sections cut at 10 μ were stained for 30 min. in a mixture of dye solution and buffer in equal parts. The following Grübler dyes were used in the concentrations indicated: 0.01% ponceau P. R. or bordeaux red (both acid); 0.005% toluidine blue or safranin (both basic). The sections were washed for 2-3 sec. in buffer of the same pH and dried by blotting in such a way as to avoid wrinkling or too complete air-drying of sections; they were dehydrated with xylol and mounted in balsam.

The pH-range of rapid change in color intensity is the isoelectric zone of the tissue element investigated; its mean value is regarded as the isoelectric point (pH). The intensity of color was estimated numerically with a color scale as follows: To a series of four test tubes were added respectively 4 ml. dye in concentrations of 0.01% \times 0.8, 0.01% \times 0.8², 0.01% \times 0.8³, etc. The basic dyes were diluted with an equal amount of alcohol. A color scale was then sketched for comparison with the depth of the tone of the stained tissue. The article should serve as a working basis for those interested in isoelectric point determinations. Tables showing results with the various buffer mixtures mentioned are given.—*J. M. Thuringer.*

SEKI, M., and KOHASHI, Y. Bestimmung des isoelektrischen Punktes der Gewebeelemente auf färberischem Wege. II. Versuche an dicht strukturierten und lipoidreichen Gebilden. *Folia Anat. Jap.*, 19, 53-7. 1940.

The colorimetric method of pH determination proved unsuitable for certain bacteria, and for the lecithin of the myelin sheath of nerves. The negative results are attributed to the dense texture of the structures and the presence of a large amount of lipoid substances.—*J. M. Thuringer.*

SEKI, M., and CHIN, K. Bestimmung des isoelektrischen Punktes der Gewebeelemente auf färberischem Wege. III. An Embryo von Huhn. *Folia Anat. Jap.*, 19, 59-64. 1940.

The isoelectric point was determined in cells of chick embryos of 3, 4, 5, 6, 7, 8, 9, 10, 12, 17, and 21 days incubation. The isoelectric point of the cells moved definitely toward the less acid side, a little more pronounced in the cytoplasm than in the nucleus. The rate of the changes was more rapid in the earlier stages than in the latter. The pH of the various cells differed very slightly in the earlier stages.—*J. M. Thuringer.*

SEKI, M., and KOHASHI, Y. Bestimmung des isoelektrischen Punktes der Gewebelemente auf färberischem Wege. IV. An Kaninchenembryo. *Folia Anat. Jap.*, 19, 65-8. 1940.

In the germ layer of the rabbit embryo the isoelectric pH-value of the cells was mesoderm > entoderm > ectoderm. The epithelial cells of the yolk sac and the allantois as well as the syncytium of the chorionic villi have a relatively high pH_i value. The isoelectric point of the ground substance of hyaline cartilage gradually moves toward the more acid side in contrast to most tissue constituents. The same may be said to a lesser extent in application to the gray and white matter of the spinal cord.—J. M. Thuringer.

SEKI, M., and CHIN, K. Bestimmung des isoelektrischen Punktes der Gewebelemente auf färberischem Wege. V. An Epithelien des oberen Luftweges und oberen Verdauungskanals. *Folia Anat. Jap.*, 19, 118-6. 1940.

The local variations in the isoelectric point of the epithelia of the upper respiratory passages and upper alimentary canal were investigated. Epithelia having great resistance toward mechanical injury such as the stratified squamous epithelium of the gums, cheek, and lip had higher isoelectric points. This was attributed to the dense texture of the structures caused by syneresis of the cell colloids; correspondingly a lower isoelectric point was shown by the stratified squamous epithelium with lesser textural density over the regions of the lymph nodules.

The isoelectric point of formalin fixed and preserved material diminishes somewhat with time, the change being greater in the cytoplasm than in the nucleus.—J. M. Thuringer.

SEKI, M., and MUKOHATA, J. Bestimmung des isoelektrischen Punktes der Gewebelemente auf färberischem Wege. VI. An Epithelien der männlichen Urogenitalorgane. *Folia Anat. Jap.*, 19, 117-20. 1940.

Formalin-fixed human material gave the following isoelectric points:

1. Epithelium of proximal convoluted tubules of kidney (characterized by abundance of basic substances) had a high isoelectric point (4.8). 2. The superficial cells of the fossa navicularis of the male urethra and the trigon of the bladder had the highest isoelectric point (4.85) of all. 3. The cells of Sertoli have a higher isoelectric point (4.70) than the spermatogonia and spermatocytes (4.60 and 4.65, respectively).—J. M. Thuringer.

STAIN TECHNOLOGY

VOLUME 16

OCTOBER, 1941

NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

NO FURTHER CERTIFICATION OF GENTIAN VIOLET

Recently the following announcement has been sent to the American stain manufacturers who have been submitting their products to the Stain Commission for Certification:

"Beginning Jan. 1, 1942, we will no longer certify any dye under the name 'gentian violet'. It must be labelled 'crystal violet', 'methyl violet 2B', or whatever it may be. After this name may be added a statement: 'Formerly sold as gentian violet', but this statement must be in small type."

Possibly a few words of explanation should be added to this statement for the benefit of those who have in the past been using one of these dyes under the name "gentian violet". This name has never had any very definite meaning, and has never been recognized in dye indexes. Biologists, however, have always been familiar with the term, as Dr. Grübler, who first attempted to standardize biological stains, continuously sold a mixture of dyes under this name. His "gentian violet" was stated to be a mixture of crystal violet and methyl violet diluted with 50% dextrin.

This product may have been quite constant in composition as long as Dr. Grübler was in charge of its preparation; but it proved very difficult for any other manufacturer to duplicate. The reason for this is clear. Altho crystal violet is a definite chemical compound (hexa-methyl pararosanilin), methyl violet is a variable mixture often containing this same hexa-methyl pararosanilin together with the penta-methyl and tetra-methyl homologs. This relationship has been repeatedly discussed in the pages of this journal, and for more detailed information the reader is referred to *Biological Stains*.¹ Enough here to state that a mixture of methyl violet and crystal violet would presumably be identical with one of the bluer shades of methyl violet, as for instance methyl violet 2B, 3B or 4B.

When the work of standardizing biological stains was begun in this

¹Conn, H. J. 1940. *Biological Stains*. Biotech Publications, Geneva, N. Y.
4th Ed. See pp. 122-6.

country, it was at first desired to eliminate the name "gentian violet" in favor of one or two of the more definite names listed in dye indexes. This suggestion, however, met with considerable opposition both on the part of users and dealers of biological stains; the former were always used to ordering "gentian violet" and the latter wanted to know how they could supply the demand if not allowed to sell a dye under that name. Finally, therefore, the Stain Commission defined "gentian violet" in the 1st edition of Biological Stains (p. 69) as "either penta-methyl or hexa-methyl pararosanilin or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B".

This has never been recognized as a satisfactory solution of the question, and in the last edition of Biological Stains the following statement is made: "The Commission does not wish to continue to give official recognition to a dye of such indefinite nature as gentian violet; and as soon as users of stains seem to be sufficiently educated in the matter, certification will be refused on any dye of this name." This time now seems to have come; accordingly the announcement to the stain companies quoted above.

Many users of stains have for some time been ordering either crystal violet or methyl violet instead of "gentian violet". For the benefit of those still using one or the other out of bottles bearing the last-mentioned label, the following information is given to guide them in what to use in the future: The National Aniline Co. and the Hartman-Leddon Co. have both been selling the same dye as either crystal violet or "gentian violet" for at least 15 years; Coleman and Bell have been selling one of the bluer methyl violets under the name "gentian violet, improved", and the same product will undoubtedly be available from them in the future labelled more definitely but with a statement added as to the name under which it was formerly sold; the European "gentian violets" when last tested were methyl violets, but none of them have been recently analyzed. This information will help a user in duplicating a batch of "gentian violet" already on hand; if further information is needed the following hint from the last edition of Biological Stains may be useful: "Users should specify crystal violet for bacteriological work, and for histological work where a deep blue-violet is required; but should order methyl violet 2B in histological procedures where a reddish shade is called for."—H. J. CONN.

PLANT-VIRUS DIFFERENTIATION BY TRYPAN-BLUE REACTIONS WITHIN INFECTED TISSUE¹

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ABSTRACT.—Trypan blue has proved effective for demonstrating the presence of certain plant viruses within infected tissues. The amorphous and crystalline inclusions which constitute cytological evidence of viruses stain proportionately. The effects produced by different viruses react differently to the stain and those inclusions which do not absorb trypan blue tend to stain with phloxine. This selective staining is the basis for using trypan blue singly and in combination with phloxine as standardized procedures for demonstrating and differentiating cytological evidence of plant viruses. These tests are very rapid and are especially applicable to temporary mounts of living tissue but permanent mounts can be made from material fixed in formalin.

It has long been known that the cells of some plant species, when infected with certain viruses, contain cell inclusions which are not found in uninfected plants of the same species. These inclusions are of many types varying from crystalline or para-crystalline structures to amorphous masses. The amorphous inclusions have been called x-bodies. These various inclusions are protein in nature and are, in part at least, the virus itself (Bawden, 1939). It is not surprising, therefore, that cytological changes brought by viruses are often just as definite and sometimes more definite than the gross morphological changes by which plant-virus diseases are usually identified. Inclusions are therefore referred to in this paper as "virus evidence".

Better methods have long been needed for investigating cytological evidence. Paraffin methods are very unsatisfactory because the process may change the relatively unstable products of virus activity and because sections seldom permit one to visualize the cell changes as a whole and relate the virus effects to the norm. In fact a critical survey of the literature indicates that examination of paraffin sections is directly responsible for the conflicting statements in literature

¹Published as Technical Paper No. 371 with the approval of the Director, Oregon Agricultural Experiment Station. Contribution of the Department of Botany in Cooperation with the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

regarding the occurrence and nature of x-bodies. Whole mounts are far more instructive than thin sections except for special cases such as phloem studies. Dr. Sheffield has combined the Feulgen reaction with the dioxan whole-mount method. This process is effective for nuclear differentiation but it does not permit proper analysis of the inclusions. Examination of living infected material with visible and ultra violet light, and frequent comparison with non-infected material is essential for a reliable interpretation of virus evidence. With this viewpoint in mind the writer has tested many vital and semivital stains to find one which would abet examination of living material and also be applicable to material fixed in neutral formalin. Trypan blue (N.A.Co. 7286) has been found specific for this purpose. The dye has an affinity for the crystalline virus elements of some inclusions and for nuclei. This coincident staining is significant since Bawden (1939) and others have shown plant viruses are chemically related to nucleoproteins. On the other hand, some viruses are associated with inclusions which are not readily stained by trypan blue. The reaction, therefore, may be used as a differential test. This paper gives directions for its use first (Test 1) for those viruses whose inclusions are readily stained and secondly (Test 2) in combination with phloxine for those viruses whose inclusions are not stained by trypan blue alone.

The application of these methods to various plant viruses has already justified the use of the term "viroplast" for inclusions formerly classified as "amorphous" or "x-bodies" since it has been possible to show there are at least ten distinct forms of these, some of which contain demonstrable crystalline elements (McWhorter, 1940). Unfortunately, these tests may affect the protoplasts and induce plasmolysis and other changes which must be constantly checked with living material before one can form a final opinion about the constitution of inclusions or other cytological evidence, even tho these may be vividly shown by the stains.

Stains and Reagents. The stains required are 0.5% and 0.05% trypan blue and 0.5% phloxine, all made up in physiological salt solution (0.85% aqueous NaCl). The effectiveness of these staining reactions depends on using a suitable detergent or wetting agent to avoid air bubbles in tissue mounts and insure uniform penetration of stains. A 1% solution of the chemically pure form of Vatsol known as O. T. 100, dissolved in hot water, has proved excellent for these and many other laboratory technics.

Preparation of Plant Material for Mounts. The surface of leaves or stems should be gently rubbed with water, or if they are difficult

to wet, they should be dipped in the detergent for about 10 seconds, then gently rubbed in water. Peelings should be made by cutting into the tissues parallel to the surface and then stripping off the piece. This can be done easily with a thin chisel knife made of razor steel which can be sharpened so that the edge will slip under the epidermis of even a thin leaf. The peelings should be immersed in the detergent for not more than 3 seconds, and then passed into a dish of physiological salt solution. Duplicate peelings should be available so that the two following tests can be started simultaneously.

Test 1—Trypan Blue Absorption. Pour 0.05% trypan blue into a watch glass and place one or more peelings in the liquid.—If virus evidence reacts positively to trypan blue, the peelings will assume a light blue tone in 15 minutes. The end-point in the staining is reached in 30 minutes. Nuclei assume a light blue color and virus evidence becomes intensely blue. Other parts of living cells do not stain appreciably, but certain tissues such as xylem will absorb the stain strongly. If the subject reacts negatively to this dye, very little color will be absorbed by living cells in 30 minutes, since only the nuclei stain. Chloroplasts remain green, a circumstance that enables one to examine readily the chlorenchyma cells in the cut ends of the peelings, since blue stained inclusions, especially crystals, contrast with the green (Plate 1, Fig. 2). Starch grains, elaioplasts, and oil globules are not stained.

It should be noted that nuclei in healthy or diseased tissue swell noticeably during the staining process. This can be circumvented by first fixing in formalin as outlined below.

Test 2—Phloxine-Trypan-Blue. Immerse the peeling in 0.5% phloxine for 3 to 8 seconds. Remove quickly and wash briskly in physiological salt solution. Transfer the peeling, which should now appear pink, into a few drops of the 0.5% trypan blue, for at least 2 to 4 minutes unless the piece of tissue is very small. Rinse and mount in physiological salt solution.—The viroplasts that do not absorb trypan blue should now appear bright pink or even dark red. In locations where the stains balance properly, the nuclei will be deep blue and the viroplasts pink or purplish, according to their ability to absorb trypan blue. Some crystalline inclusions remain unstained. Chloroplasts remain green and virus evidence becomes conspicuous even in chlorenchymatous cells. The effect is not unlike a Giemsa blood stain except that chlorophyll adds a green coloration to the array. The chief purpose of Test 2 is to stain differentially and to make readily discernible those viroplasts that do not absorb trypan blue when used as directed in Test 1.

Special Cases and Variations. Material which cannot be examined at once may be fixed in 5% formalin. As fixation progresses, the staining reactions become less and less effective. Protein cell contents become yellow in formalin and are thereby made more visible, but this coloration is very unsatisfactory for direct examination or photographic rendering. It therefore seemed desirable to the author to treat the fixed material with some reagent that would restore the staining properties of the tissue. At the suggestion of Dr. J. R. Haag, Department of Chemistry, Oregon State College, citric acid was tried for this purpose. Fortunately, this reagent makes the tissue again susceptible to differential staining with trypan blue. The procedure for formalin-fixed material is: (1) to prepare the peelings from the fixed material, (2) to place them directly into a 10% solution of citric acid for 1 to 6 hours, (3) to place them in the stain.

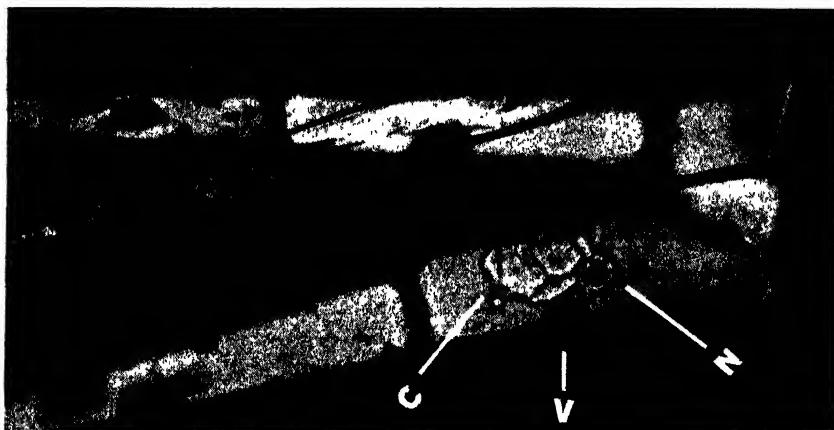


Fig. 1. Differentiation by Test 2 of tobacco mosaic virus evidence in *Lycopersicum trichome*. C, hexagonal crystal; V, compacted-alveolar viroplast; N, nucleus of the cell.

The concentrated dye may be used as directed below but 30 minutes in 0.05% usually gives an excellent preparation.

The concentrated 0.5% solution of trypan blue may be used to good advantage to stain material fixed in formalin when attempting Test 1. The average time required is 4 minutes. Moreover, living material known to contain virus evidence that reacts positively to trypan blue, may be quickly stained in the concentrated solution, the time being reduced to approximately 3 minutes.

Peelings from pubescent leaves make beautiful preparations by the method used in Test 2 if the cover is firmly flattened down and the tissue left in the trypan blue stain during examination. Since the



Plate I

1. Trypan blue demonstration of virus evidence after formalin fixation of peeling from lily leaf infected with latent virus complex. F, long curved fibrillae; V, fibrillate-granular viroplast; N, nucleus. $\times 350$.
2. Demonstration of isometric crystals in Bean Virus 2 infection by Test 1 method applied to living leaf material. The proportionate stain absorption renders the large crystal darkest, the rosette of crystals in nucleus lighter, while the disintegrating plastid remains unstained. $\times 3000$.
3. Test 1 applied to peeling from living tulip leaf infected with tulip virus 1. Note proportionate stain absorption. N, nucleus; CV, compacted viroplast; PV, particulate-reticule viroplast. $\times 400$.

stain does not color the cell walls, the effect approximates a deep blue darkfield in which the trichomes stand out as brilliantly illuminated objects. (Fig. 1.) The progress of Test 2 can be followed in trypan blue mounts and the tissue removed when a desirable endpoint has been reached in the epidermal cells.

Permanent mounts can be made from formalin-fixed material by following the dioxan whole-mount technic (McWhorter and Weier, 1936). They should be deeply stained in an aqueous solution of trypan blue before dehydrating them. The dioxan process yields mounts with a refractive index favorable for examination of cell parts.

Illustrations and Suggestions. The illustrations accompanying this article show selective staining characteristic of these tests. The sharp staining of virus evidence without staining other cell contents, except nuclei, facilitates the detection of viruses by utilizing cytological clues. For example, Test 1 was directly responsible for the discovery by the author (1941) of crystals which average less than a micron in diameter in green chlorenchymatous cells of leaves infected with certain legume viruses. (Plate 1, fig. 2.) It is hoped that selective staining may become of specific use in the classification of plant viruses.

The procedures seem almost "fool proof" but there are, of course, chances for unexpected variations and for misinterpretation. The following suggestions relate to points where variations may occur. The detergent must be used for a very short time (seconds only) to prevent too rapid disintegration of protoplasts. No interpretation is possible until the nuclei have been well stained. Altho fats do not absorb trypan blue, it is well to substantiate one's conclusions by applying selective fat stains so that unusual elaioplasts cannot be mistaken for protoplasmic formations related to viruses. Formalin fixation involves coagulation that may modify the original structure of viroplasts. Continual checking with carefully prepared living mounts is always advisable.

- (1) BAWDEN, F. C. 1939. Plant Viruses and Virus Diseases. 272 pp. Chron. Bot. Co., Leiden, Holland. (Contains an excellent discussion of the relation of inclusions to viruses.)
- (2) McWHORTER, F. P., and WEIER, ELLIOT. 1936. Possible uses of dioxan in botanical microtechnic. *Stain Techn.*, 11, 107-17.
- (3) McWHORTER, F. P. 1940. Separation of tulip 1 virus from lily-latent by cytological methods. *Phytopath.*, 30, 788.
- (4) McWHORTER, F. P. 1941. Isometric crystals produced by *Pisum* virus 2 and *Phaseolus* virus 2. *Phytopathology* 31, 760-1.

THE GERMINATION AND STAINING OF BASIDIA IN GYMNOSPORANGIUM

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ABSTRACT.—A procedure is described for germinating and staining rust teliospores on the slide. The spores are germinated on slides in a damp chamber, about 3 hours being required for the production of sporidia. The material is killed by inverting slides over osmic acid fumes for a few minutes. Germinated spores are then allowed to dry on the slide, thus becoming fixed to the slide in a gelatin produced by the breaking down of their own stalks during germination. No other fixative is required. Material must be thoroly dehydrated in the alcohols (one or more hours in each of the higher alcohols); returned to water; mordanted for 2-3 hours in 4% iron alum; stained for 2-3 hours in 0.5% aqueous solution of Heidenhain's hematoxylin; destained in 2% iron alum. The material is passed back thru the alcohols and mixtures of xylol and absolute alcohol (1:2, 1:1, 2:1) to xylol and mounted in balsam. The method is particularly satisfactory for the *Gymnosporangium* rusts, which have telia very readily gelatinized. The details of germination are preserved intact, as in nature, and many details of nuclear division are excellent.

In the study of germinating teliospores of *Gymnosporangium clavipes*, a rust with the telial phase on red cedar, a very convenient and successful method has been devised for germinating the teliospores and staining the nuclei in the division stages in the basidia and sporidia. By this method, which takes advantage of the natural gelatin in the teliospore stalks to fix the material to the slide, one of the greatest difficulties in handling germinating basidia in this group of rusts is overcome, i.e., the basidia are not torn away from the teliospores and the basidiospores are not broken off from the basidia. Teliospores of other rusts were germinated in drops of water on slides almost a hundred years ago, but (so far as the writer knows) this is the first time anyone has used the natural gelatin in the telial stalks to glue the germinating spores on the slide.

The teliospores are collected in early spring on cedar branches, where they occur as bright orange pustules on slightly swollen areas

¹The writer is grateful to Professor J. N. Couch for helpful suggestions during this work.

of the branches. It is necessary that the rust be collected while still dry, and before the rains have caused a gelatinization of the telia. These spores will give good germination results for at least two weeks after being brought into the laboratory.

On a clean slide a single drop of distilled water is placed. With a needle a small mass of teliospores is teased out of a mature pustule and placed in the drop. The spores are then spread out with the needle so that they do not remain together in one mass, but lie in a more or less even layer. The slide is now transferred to a damp chamber for germination.

The water in which the teliospores are placed soon causes the spore stalks to gelatinize so that the spores come to lie in a gelatinous matrix on the slide. Spores germinate well at room temperature. After about three hours in the damp chamber they are usually ready to be removed. An examination under the microscope will show the presence of abundant basidia in the process of producing sporidia.

Before the procedure is continued, the germinating spores should be stirred thoroly with a needle in order to make the basidia lie on their sides. Otherwise the sterigmata with their sporidia will point straight upwards and will be unsatisfactory for study. The stirring process does not appear to injure the basidia in any way and does not dislodge the sporidia unless they are ready to be discharged. If the spores are still too closely massed together, they should be spread out into a thinner film, with more water added to facilitate this, if necessary. The natural gelatin produced by the spore stalks is the only substance required by this procedure for fixing the spores onto the slide.

The material is now ready to be killed. It is desirable to use a quick killing agent and one which does not require the slide to stand in liquid medium; otherwise the spores might be quickly dislodged from the slide. A killing chamber employing fumes of osmic acid is most effective. A watch glass is placed in a Petri dish and a few drops of 1-2% osmic acid are put into the watch glass. The slide, with the material on it still moist, is inverted over the osmic acid in the watch glass, and the dish is closed. The fumes of the acid kill the germinating spores quickly and the slide may be removed after 4-5 minutes in the killing chamber.

The slides are now placed out in the open and allowed to dry. The drying is accomplished usually within half an hour, the time varying with the amount of moisture used. The gelatin hardens and fixes the spores onto the slides. This does not harm the basidia, for the gelatinous film around them protects them from desiccation.



Plate 1. Photomicrographs of germinating teliospores of *Gymnosporangium clavipes*.

Fig. 1. Telophases of the second nuclear division in the basidium. $\times 1000$.

Fig. 2. Anaphase of the first nuclear division in the basidium. Figure also shows a discharged binucleate sporidium. $\times 1000$.

Fig. 3. Basidia, one of which is producing sporidia. $\times 500$.

Fig. 4. Basidium producing sporidia, with nuclei passing into the sporidia. $\times 500$.

This process occurs normally in nature between rainy periods. The material may even be left for several days in this condition without any obvious ill effects.

The procedure following the osmic acid treatment lays emphasis on thoro dehydration, subsequent to employing Heidenhain's iron alum hematoxylin staining technic. It will be noticed that slides are left for only short periods in water and the lower alcohols. This is to prevent the spores from becoming dislodged. After passing thru 50% alcohol, however, there is little danger of losing much of the material thruout the remainder of the procedure.

The necessity for thoro dehydration is apparent from results obtained by varying the length of time in the alcohols. When the slides were run up thru the alcohols at 10-15 minute intervals, there were left in the cytoplasm certain stainable inclusions which obscured all nuclear details after treatment with iron alum hematoxylin. What these inclusions are is not known to the author. They are removed by leaving the slides for longer periods in the higher alcohols.

The steps following the osmic acid treatment may be listed as follows:

1. Water for 2 or 3 min.
2. 10% alcohol for 15 min.
3. 20%, 30%, and 40% alcohols for 30 min. each.
4. 50%, 60%, 70%, and 80% alcohols for 1 hr. each.
5. 95% alcohol for at least 3 hr. Slides may be left overnight.
6. Absolute alcohol for 1 hr.
7. Return thru the alcohols to water at 15-min. intervals, bleaching the slides in 50% alcohol containing a little hydrogen peroxide (10 cc. H₂O₂ to 100 cc. 50% alcohol).
8. Water for 20-30 min., changing several times.
9. 4% iron alum for 2-3 hr.
10. Water for 15 min., changing several times.
11. Heidenhain's hematoxylin (0.5% aqueous solution) for 2-3 hr. A longer time is undesirable, as it makes destaining of the cytoplasm more difficult.
12. Water for 30 min., changing several times.
13. Dehydrate, passing thru the alcohols at 15-min. intervals.
14. Mixtures of absolute alcohol and xylol in ratios of 2:1, 1:1, 1:2 for 10 min. each.
15. Xylol for 30 min.
16. Mount in balsam.

The nuclei should now be stained so that they stand out distinctly in various stages of their history from early prophase in the basidium

to their passage into and division in the sporidia. They show up as black figures against a light cytoplasmic background. We have made a large number of slides showing distinct spindles during nuclear division in the basidia and in the sporidia. The technic is not one designed to show up individual chromosomes well enough to be certain of their form and number, but definite chromosomal units can often be seen on the spindles.

Figures 1 and 2 show the nuclei in the basidia in the process of meiotic division. The former illustrates the second meiotic division in telophase, and spindle fibers can be seen between the daughter nuclei. Figure 2 shows the anaphase of the first meiotic division. Figures 3 and 4 show basidia giving rise to sporidia. In figure 4 two nuclei were caught in the process of passing thru the sterigmata into the sporidia.

In several ways this technic is decidedly superior to the more complicated methods previously used in making basidial preparations of this group of rusts. By taking advantage of the natural gelatin produced by the stalks of the teliospores to fix the material to the slide it is possible to kill and stain material exactly in the stages desired. Furthermore, by avoiding a liquid killing agent followed by washing, the basidia remain attached to the teliospores and the sporidia remain attached to the basidia. Finally the injury to the protoplasmic structures is so slight that with appropriate staining excellent nuclear figures are obtained.

PASTERNACK'S PARAFFIN METHOD MODIFIED FOR PLANT TISSUE¹

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ABSTRACT.—This method for preparing paraffin sections of plant material is a modification of Pasternack's one-hour method for animal tissues. Fixation in Randolph's CRAF fixative is hastened by heat and increased vapor pressure obtained by the use of screw top vials. Dehydration with Zirkle's butyl alcohol series likewise is hastened in the same manner. The rapid penetration of paraffin by the use of $\frac{1}{2}$ paraffin and $\frac{1}{2}$ butyl alcohol in heated screw top vials shortens the embedding process. Sections are held on the slide thru staining by albumen fixative and a coating of 0.2% celloidin in absolute alcohol and ether. Good penetration with freedom from shrinkage or distortion is obtained and root tip chromosome counts can be made in approximately 3 hours.

Pasternack² has devised a one-hour technic for the preparation of paraffin sections of animal tissues. A combination of Pasternack's technic with existing conventional plant methods has yielded a schedule which in our work with the *Bromeliaceae* enables us to obtain root tip chromosome counts from paraffin sections in approximately 3 hours. This method combines good penetration with freedom from shrinkage or distortion of the tissues and the chromosomes are well separated.

The time-saving features of this schedule over the conventional methods used in plant cytology make it seem worth while to call it to the attention of other investigators.

Rapid penetration of the root tip tissues is obtained by heating the reagents as indicated in the schedule below:

1. *Fixing.* Heat root tips to 80° C. in a well stoppered or screw top vial containing Randolph's CRAF fixative (solution A: chromic acid 1 g., glacial acetic acid 7 cc., water 92 cc.; solution B: neutral formalin 30 cc., water 70 cc.) and keep at this temperature for 10

¹Published with the approval of the Acting Director as Miscellaneous Paper No. 35 of the Pineapple Experiment Station, University of Hawaii.

²Pasternack, Joseph G. A reliable one-hour method for the preparation of paraffin sections of tissues. Amer. J. Clin. Path., Tech. Suppl., 4, 8-18. 1940.

minutes. Wash in several changes of warm water, 15 minutes minimum. Blot dry and wipe off any adhering soil particles.

2. *Dehydration*. Run the root tips thru Zirkle's³ butyl alcohol series.⁴ (A 5-minute treatment in each butyl alcohol step is sufficient with the material used in this experiment.)

- A. Butyl I (10 cc. *N* butyl, 43.5 cc. 95% alcohol, 46.5 cc. water).
- B. Butyl II (20 cc. *N* butyl, 54.0 cc. 95% alcohol, 26.0 cc. water).
- C. Butyl III (35 cc. *N* butyl, 54.0 cc. 95% alcohol, 11.0 cc. water).
- D. Butyl IV (55 cc. *N* butyl, 43.5 cc. 95% alcohol, 1.5 cc. water).
- E. Butyl V (75 cc. *N* butyl, 25.0 cc. 95% alcohol).
- F. Normal butyl I.
- G. Normal butyl II.
- H. Normal butyl and paraffin (1:1), 10 minutes.

3. *Embedding*. Place material in paraffin in a paraffin oven at 60° C. for a minimum of 15 minutes, preferably in a vacuum container; if convenient, leave in the oven during the lunch hour. Embed in paraffin and immediately place in ice water. Cut sections 5 to 10 μ .

4. *Mounting on slide*. Affix to slide with Meyer's fixative. Wipe excess water from back and edges of slide. Put on hot plate, 50° C. for 5 minutes. After melting, remove paraffin with xylene from a dropping bottle. Before the xylene evaporates, cover the section with 0.2% celloidin in absolute alcohol and ether in the proportion 1:1, using the ordinary dropping bottle. Drain off excess fluid and blow to hasten evaporation. Immerse the slide immediately in water to harden the celloidin. If done with care no undue shrinkage results from these steps. The extremely dilute concentration of celloidin leaves no trace on the finished slide.

5. *Staining*. Stain 3-10 minutes with a 1% aqueous gentian violet (Newton), followed by the iodine, alcohol, clove oil, xylol balsam series.

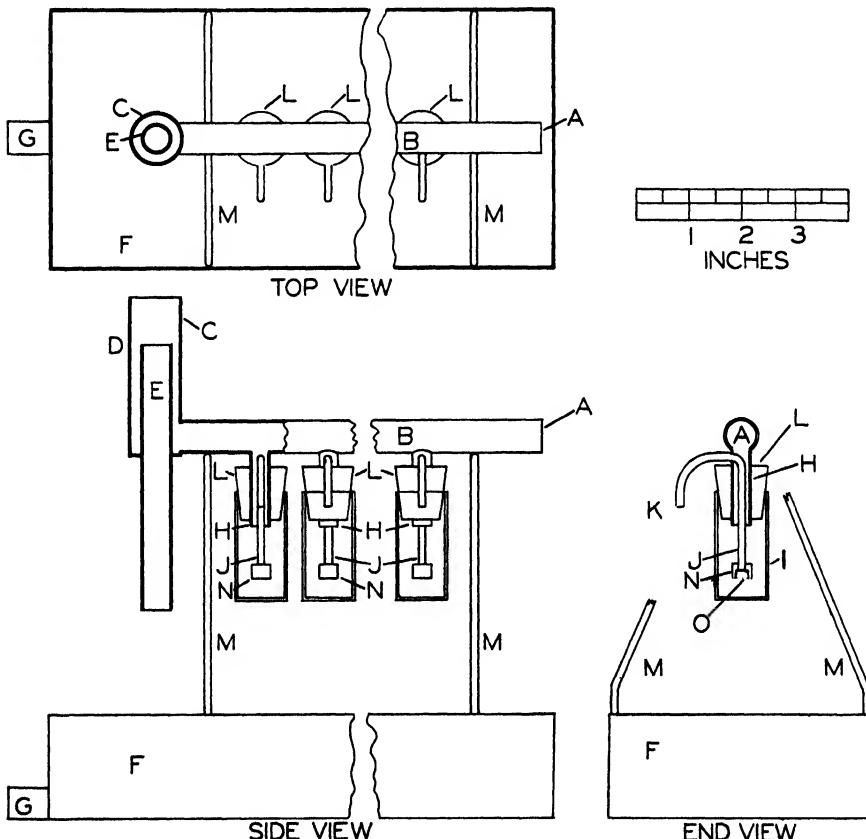
³Zirkle, Conway. The use of *N*-butyl alcohol in dehydrating woody tissue for paraffin embedding. *Science*, 71, 103-4. 1930.

⁴Reagents are preheated to 56-60° C. Increased vapor pressure is obtained by screwing the lids on the vials as soon as the roots are inserted; the use of 6 to 8 cc. reagent in 20 cc. vials is recommended.

AN APPARATUS FOR WASHING TISSUE

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The apparatus shown below, which can be constructed in the ordinary departmental shop, has been found useful for washing large numbers of individual specimens preparatory to making histological



sections and is particularly applicable in handling successive portions of relatively large structures which are to be studied in series.

In operation, water from the laboratory faucet enters a horizontal brass tube **B**, at **A**, by means of a hose not shown in the figure. The faucet is adjusted so that water fills the tube **B**, and the vertical tube **C** where a considerable portion overflows at **D** thru tube **E** into the

pan F and out to the drain thru tube G and a hose not shown in the figure. This arrangement provides a constant head of water regardless of small variations in the tap water pressure. The specimens to be washed are placed in glass vials connected to the apparatus by the rubber stoppers L which normally remain attached to the tubes H. The way in which the water flows while washing the specimens can best be seen by examining the drawing at the lower right of Fig. 1, labeled "End View". Part of the water in tube A is diverted thru H into the glass vial I, then up thru the screen O, which is soldered to an enlarged opening N, which in turn is soldered to the eighth inch copper tube J. The water leaves this tube at K, falls into the tray F and finally leaves at G with the water from the other vials and that from E. The rods M attach the tube B to the pan F.

In the figure only three vials are shown altho as many as may be desired may be attached to the tube B. In the model used by the authors there were twenty vials. The entire apparatus is made of brass with the exception of the rubber stoppers L, the copper tube J and the glass vials I. All joints except those made by the rubber stoppers, are soldered. The drawing has been made to the scale shown at the top right and for the sake of clearness the vertical tubes C and E have been omitted from the end view.

The best way to assemble the apparatus is as follows: (1) drill the holes for tubes H, in tube B; (2) solder tubes H in place; (3) drill holes in tubes H for tubes J making them somewhat larger than the actual diameter of the latter; (4) the eighth inch copper tubes J are soft and easily bent so that, starting with straight tubes, one end is inserted in the appropriate hole in H, and with a combination of pushing and bending it is pushed inside H and finally bent with the fingers to the form shown in the end view; (5) the joint between J and H is then soldered. No particular order of assembly or instructions are needed for putting together the remainder of the apparatus.

PERMANENT STAINED PREPARATIONS OF THICK BLOOD FILMS

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ABSTRACT.—Fixing thick films in alcoholic solution of dye after the usual staining-and-laking procedure preserves the appearance of parasites and blood elements very similar to that of the usual thick films (not fixed) for the diagnosis of malaria and relapsing fever.

Procedure recommended: Films are stained and laked for 15 minutes in diluted Giemsa—1 to 3 drops of stock solution (0.4 g. in 60 ml. equal parts absolute methyl alcohol and glycerin) per ml. distilled water; rinsed in water and allowed to dry. They are then immersed in, or flooded with, May-Grünwald's stain (0.5% in absolute methyl alcohol) for 30 seconds, rinsed in water and allowed to dry. Solutions of MacNeal's tetrachrome stain in methyl alcohol and glycerin may be substituted for Giemsa and a solution in methyl alcohol may be substituted for May-Grünwald. With slight modification of the procedure, both thick and thin films on the same slide may be stained together.

Films stained and fixed as described, and mounted in Diaphane, have shown no evidence of fading in 3 years.

The thick blood film technic has been very useful in the diagnosis of certain infectious diseases, particularly malaria and relapsing fever. Unfortunately, films stained by the usual procedure (Barber and Komp, 1929), in which laking of the red cells and staining the remaining blood elements and parasites is accomplished by diluted Giemsa stain, are not permanent and one can count on fading within a few months depending on climate and other conditions. Covering films with liquid petrolatum or with petroleum jelly to prevent fading is often inconvenient.

In order to attain fixed preparations with staining properties similar to those of films stained in the usual manner, a number of laking and fixing agents were tried with dried thick films of blood containing the parasites of tertian malaria (*Plasmodium vivax*) or malignant malaria (*P. falciparum*). Staining with diluted Giemsa (or other blood stains) was unsatisfactory in one or more respects following treatment with: (1) 0.5 to 1% glacial acetic acid and 1% formalin (Ruge, 1903); (2) 2% glacial acetic acid and 0.4% tartaric acid—

also followed by methyl alcohol (Knowles, 1931); (3) acid alcohol—0.5 to 3% hydrochloric acid (James, 1911) or 5% acetic acid (Henson, 1913); (4) diluted ethyl alcohol, 10% to 70% (Vaillant, 1919); (5) acetone, water, alcohol (Bramachari and Sen, 1928). Similarly poor staining—whether by Giemsa or other technic—followed treatment of the films with simple laking agents such as distilled water, saponin in water or physiological saline, 5% glycerin and 5% methyl alcohol in water, ethyl acetate, etc. The characteristic staining of blood elements and parasites observed in films laked and stained in diluted Giemsa was altered unfavorably by *previous* treatment with any of the laking or fixing agents.

Fixation of films with alcoholic solution of dye *after* the usual staining (and laking) was found to yield satisfactory results, even tho the films were stained slightly different. A good many comparative trials were made with a number of blood stains in methyl alcohol and in equal parts of alcohol and glycerin. Solutions of Giemsa,¹ MacNeal's tetrachrome,² Wright,³ azure-II-eosine,⁴ May-Grünwald⁵ and several non-commercial samples of dye were prepared in absolute methyl alcohol (0.15 g. dry stain per 60 ml., May-Grünwald 0.3 g. per 60 ml.) and in equal parts of absolute methyl alcohol and glycerin (0.4 g. dry stain per 60 ml. solvent) on several occasions and tested on blood films from various sources. Dried thick films were first stained-and-laked according to the usual technic—i.e., immersed in diluted alcohol-glycerin stain (1-3 drops of stock solution per ml. distilled water⁶) for fifteen minutes, very gently rinsed in distilled water and allowed to dry. Films stained by a given dye were then fixed with one or another of the alcoholic solutions of the several samples, either by immersing the slide or flooding it for a minimum period, rinsed in water and allowed to dry. Most satisfactory results were obtained with a period of fixation of about 30 seconds.

Most of the dyes stained the parasites at least well enough so that they could be recognized. Since Roe, Lillie and Wilcox (1940) have determined the particular dye components and their proportions which stain malarial parasites most satisfactorily, a detailed report of the relative merits of the samples used in this work is not needed. It may be stated, however, that the chief difficulty with

¹Grübler's, NGe-1, NGe-2, NGe-3, NGe-5, CGe-1, LGe-1.

²NMn-9, three samples.

³NWr-12, CWr-11.

⁴Nat. Aniline & Chem. Co. No. 8366.

-- ⁵Nat. Aniline & Chem. Co. No. 7598; Hartman-Leddon Co.

⁶Buffer is unnecessary for thick films if the salt content of the distilled water is properly low.

certain samples (especially Grübler's) was the darkly stained background of the film. And, contrary to expectation, two samples of May-Grünwald stained the parasites well in thick films, but poorly in fixed thin films.

Fixation with alcoholic solution of dye, after the usual staining, yields satisfactory preparations provided it does not destain the parasites or blood elements. Hence the limited period for fixation and the importance of saturated alcoholic solution of the stain used for this purpose. Irrespective of the type of stain used first, fixation with alcoholic solutions of May-Grünwald and of Tetrachrome usually gave somewhat better results than with alcoholic solutions of Giemsa, azure-II-eosine and Wright. To list all the details of results of the many combinations of stains and fixations with the samples of dyes used in this study would probably defeat the purpose of presenting a simple, reliable method. However, on the basis of these comparative tests a few definite procedures can be recommended: (1) staining-and-laking with Giemsa (which is most commonly used for thick films), followed by fixation with May-Grünwald (0.5% in methyl alcohol); (2) staining-and-laking in an alcohol-glycerin solution of tetrachrome (which has yielded results as good as Giemsa), followed by fixation with alcoholic solution of the same dye; (3) slight modification of the procedure for use with slides which have both thick and thin films. Description of the procedures follows.

1. Thick films, prepared by spreading one large drop of blood on a slide over an area of about 1 cm. diameter and allowing it to dry, are immersed in diluted Giemsa stain—1 to 3 drops of stock solution (0.4 g. in 60 ml. equal parts absolute methyl alcohol and glycerin) per ml. distilled water for 15 minutes.⁷ The film is then gently dipped into distilled water momentarily and allowed to dry. Fixation is accomplished by immersing the film or flooding it with May-Grünwald (0.5% in absolute methyl alcohol) for 30 seconds. The film is rinsed in distilled water and allowed to dry.

2. The dried thick film is immersed in diluted alcohol-glycerin solution of tetrachrome—1 to 3 drops of stock solution (0.4 g. in 60 ml. equal parts absolute methyl alcohol and glycerin)—for 15 minutes, dipped in distilled water and allowed to dry. The film is fixed for 30 seconds with alcoholic solution of the same dye (0.15 g. in 60 ml. absolute methyl alcohol), rinsed in distilled water and allowed to dry.

3. Thick and thin films of the same blood are frequently spread on

⁷Altho many authors specify 30 minutes or more, the writer has found that films may be satisfactorily stained and laked within as short a period as 5 to 10 minutes.

the same slide in diagnostic or survey work. The films on such a slide can be stained separately if a line of paraffin is painted between the films. A more convenient method is to stain the two films on the one slide as follows: slides are placed vertically in just enough diluted Giemsa stain to cover the thick films without coming into contact with the thin films, allowed to remain for 15 minutes, dipped momentarily in distilled water (without allowing the water to come in contact with the thin films), and allowed to dry. The slides are placed (individually) in a vial containing sufficient alcoholic solution of May-Grünwald to cover both films for 30 seconds and rinsed very quickly (incompletely) in water and then placed in diluted Giemsa in a Coplin jar for 15 minutes. The preparations are washed briefly by running distilled water into the jar (to avoid precipitate on the films) and allowed to dry. Alcohol-glycerin solution of tetrachrome may be substituted for Giemsa, and alcoholic solution of tetrachrome for May-Grünwald. The last treatment of the thick film with diluted Giemsa apparently does not alter the result in any respect.

By observing the ordinary precautions of preparing blood films as, e.g., the use of clean, grease-free slides of good grade (minimum free alkali), and by using satisfactory grades of stains (certified, if possible) and solvents, one can obtain very good preparations which are "permanent". Mounting stained blood films in Diaphane⁸ reduces the tendency to fade due to condensation on the slides which is especially troublesome in a humid climate. (Caution: temperatures above 40° C. are likely to cause fading. Balsam invariably causes stained blood smears to fade.) The writer has thick-and-thin films stained by the third method described above, mounted in Diaphane, which have not shown any tendency to fade in 3 years.

In appearance the thick films prepared by any of the three procedures are quite similar and differ from the usual stained thick film, which is not fixed, only in minor respects. In satisfactory preparations, the red (chromatin) and blue (cytoplasm) stains of malarial parasites are retained undiminished and well defined, and the blood elements appear about the same. The chief difference from the usual "unfixed" thick film is the lighter, somewhat violet background in the fixed films. Apparently the short process of fixing removes the basophilia, or polychromatophilia, a fact which is often very noticeable in thick films stained in the usual manner. In fact, Barber and Olinger (1931) were able to estimate the grade of anemia by the density of the "blue clouds" in such films.

The same procedures were tested with rat's blood containing the

⁸Will Corp., Rochester, N. Y.

spirochaetes of relapsing fever (*Borrelia novyi*)⁹ and with dog's blood containing the microfilaria of dog heart worm (*Dirofilaria immitis*). The spirochaetes appeared the same as in ordinary thick films, but were rather more easily recognized because of the somewhat clearer background. In dog's blood the microfilariae took a more intense and differential stain and, again, the background was lighter than in films not fixed.

The special value of permanent preparations of stained thick blood films would seem to be (1) for certain research purposes as, e.g., counting parasites, and (2) for use in instruction relative to the importance of thick films in the diagnosis of certain diseases and the appearance of parasites in such preparations. For experienced observers, the thick blood film offers the most sensitive, the most certain, and the simplest means for the diagnosis of malaria and relapsing fever. The fact that it is barely appreciated by the many individuals who are not specialists but frequently have occasion to use it, is undoubtedly due, in part, to the lack or paucity of suitable material for instruction. For this reason the author sought a method which would preserve, insofar as possible, the appearance of the thick film stained in the usual manner for diagnostic purposes, and hopes that the procedures described here will contribute towards a more general acquaintance of students, practitioners and investigators with the appearance of parasites in such preparations. As the technic is simple and has been fairly well standardized by Barber and Komp (1929) and by Roe, Lillie and Wilcox (1940), while the stains used for this purpose are certified by the Commission on Standardization of Biological Stains there is every reason to look forward to a greater appreciation and wider use of the thick film.

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⁹The writer is indebted to Dr. Vernon Schuhardt of the Department of Bacteriology at the University of Texas, Austin, Texas, for supplying this material.

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PREPARING PERMANENT DEEP CHAMBER MOUNTS OF VARIABLE DIMENSIONS

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ABSTRACT.—A rapid and simple method for the preparation of permanent deep chamber mounts of variable size, shape and depth is described. The chamber sides are made with aluminum wire bent on a form of the proper size and shape. Aluminum wire is nicely adapted for use due to its pliability and clean appearance. It undergoes no apparent change in contact with the mounting medium. Depth of the chambers is determined from the gauge of the wire. Clarite, used as the mounting medium, is prepared as a thick solution in toluol (70-75% of clarite by weight). The resulting preparations are crystal clear, colorless, and according to the work of other investigators, do not become acid with age.

The need for a simple and rapid method of preparing a large number of permanent deep chamber mounts of cleared thick sections of tissue prompted the development of the technic described below.

Numerous methods have been used for making mounting chambers for such things as small whole specimens and thick sections. Rings punched from various plastics, thick hollow chamber slides, covers supported by small broken bits of glass, slides with moats built up with rings of varnish or cements, have all been advocated and used for a long time. Some of the methods such as the hollow-chamber slides and commercially made mounting rings are quite expensive. Other technics produce excellent results but are tedious and slow.

The mounting medium used to fill such chambers has nearly always been Canada balsam or gum damar. It is common knowledge that Canada balsam, a deep yellow to begin with, darkens further on aging. Gum damar, while somewhat paler, also darkens. Either of these resins in a deep chamber produces an undesirable color. While it is true that this color makes no appreciable difference in the visibility of the preparation (this being mainly a function of the refractive index of the mounting medium compared to that of the tissue), it is obvious that a colorless mounting medium is technically more desirable, provided it meets other necessary requirements.

In the past four or five years a number of synthetic materials have been suggested for use as mounting media. Skiles and Georgi (1937) discussed the use of two synthetic resins as mounting media. These

resins, "Vinylite" and "Pontalite" (Lucite), were used for bacterial slide mounts and permanent mounts of moulds. They believed that these resins were superior to Canada balsam. Richards and Smith (1938), however, found Lucite unsatisfactory when used under a cover glass. Certain stains showed considerable fading and air bubbles appeared as the preparations dried. Kirkpatrick and Lendrum (1939) reported successful results with distrene 80, a synthetic resin to which they added tricresylphosphate to act as a plasticizer in reducing shrinkage from drying. This material was stated to be water-clear in xylol solution, of a constant pH, and was claimed not to cause fading of delicate stains. Isobutyl methacrylate, recently suggested as another synthetic material suitable for use as a mounting medium (O'Brien and Hance, 1940), has had certain objections raised against it by Hamilton (1940) and Groat (1940) who stated that it caused fading of stains, distortion with drying and that the refractive index (1.477) was too low for most purposes.

Groat (1939) gave the first account of the use of clarite and clarite X as mounting media. He found them superior to Canada balsam and gum damar in all respects. His paper should be referred to for a detailed description of the properties of these substances.

Little mention has been made of the practicability of using such synthetic resins for permanent deep cell mounts. Mohr and Wehrle (1940) briefly mentioned the superiority of clarite over Canada balsam for whole mounts of flukes. Smith (1940) stated that in making whole mounts of thick objects clarite had to be used in dilute solutions (60%) and showed the serious drawback of needing frequent "fill-ins" while such preparations were seasoning. Thick solutions of clarite in toluol have been found entirely satisfactory for use in the deep chambers described in this paper.

In the past practically all deep mounts were prepared in a round chamber utilizing a circular cover slip. Such covers are very much more expensive than square ones. This fact becomes a disadvantage if many specimens are to be mounted. The depth of chambers has, in most cases, apparently been a matter of guesswork. However, the possibility of using a high power objective in the examination of thick sections depends on having no excessive depth to the chamber. The technic to be described allows the use of square, rectangular or round covers of any desired size, together with the precise regulation of the depth of the chamber.

MATERIALS AND METHOD

The deep chamber preparations are made by placing a ring or square of aluminum wire of the desired thickness on the slide, placing

the object to be mounted within its confines, pouring in the proper amount of thick clarite and carefully applying a cover glass. Cementing the wire to the slide is unnecessary as a thick solution of clarite does this neatly and with little leakage, provided the wire is flat on the slide.

Aluminum wire may be obtained in the following gauges:

| Gauge (B. & S.) | Diameter in millimeters (nearest 1/100 mm.) |
|--------------------|---------------------------------------------------|
| 14 | 1.63 |
| 16 | 1.29 |
| 18 | 1.02 |
| 20 | .81 |
| 22 | .64 |
| 24 | .51 |

From this table the depth of a chamber can be determined by using wire of proper gauge. Wire rings are most easily made. Square wire frames are a little more difficult to shape perfectly but with practice this can be done.

Wire rings. A metal or hardwood cylinder of the proper size, i.e., smaller than the cover slip diameter by two times the thickness of the wire, is clamped in a vise and a number of turns of wire wound closely and tightly around it near one end. This coil is carefully slid off and with cartilage shears (or some similar cutting instrument) a cut is made along one side of the coil, crossing all the turns of wire. This produces a number of exactly similar rings. These are then shaped individually in a small, smooth-faced vise so that they will lie flat on a slide.

Wire squares. The procedure for making square wire frames is identical to that for rings, except that a square metal shaft is used. For a perfect fit the size of the shaft is again the width of the cover slip minus two times the diameter of the wire.

When the wire is centered on the slide the chamber is ready for use. The tissue is transferred from toluol into the chamber and a thick syrupy solution of clarite in toluol (70% or a little more) is poured in to fill the chamber completely. Any trapped air under the tissue is teased out and the cover slip applied. The objection raised by Smith (1940) that rapid crust formation when using toluol solutions of clarite interferes with the proper application of cover slips to ordinary slides has found an answer in the deep chambers. Crusting over does occur in a few seconds time at room temperature. If the cover slip is lowered slowly, however, starting at one edge of the

chamber, the crust redissolves rapidly as the glass touches it and no air bubbles will be trapped. Apparently the exclusion of air allows toluol from below the surface to dissolve the crust.

Drying temperatures of 38° C. over a period of two months and 56° C. for a period of three weeks have caused no cracking or retraction of the clarite in the chambers. Exposure to sunlight daily for seven days has not damaged the mounts. Exposure to heat in a carbon arc microprojector for periods of twenty minutes did not alter the appearance of the clarite in the chambers.

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ACETIC-ORCEIN: A NEW STAIN-FIXATIVE FOR CHROMOSOMES

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ABSTRACT.—A new stain-fixative method for chromosomes, namely acetic-orcein, is described, which gives results that are equally good in fresh and permanent preparations. A 45% acetic and 1% orcein content is recommended as a standard solution. For salivary glands of *Drosophila* a 2% stain gives the best results, and with the two species *D. melanogaster* and *D. miranda* the acetic strength has been raised to 70% with advantage. The addition of chloroform proves necessary for hardening in species of *Sciara*. Acetic-orcein is equally good for rapid chromosome counts. For root tips the addition of 1 cc. of N HCl solution to 10 cc. of the standard solution together with gentle heating of the tissues in a drop of the mixture assists in the softening and separation of cells necessary for chromosome study. Orcein can also be used successfully in other combinations such as acetic-propionic or acetic-lactic. The latter is useful for making preparations that do not require ringing. Preparations so made keep from 7 to 14 days.

VALUE OF ORCEIN

The aceto-carmine method now so generally used for chromosome study has two shortcomings. It is not particularly selective, especially for small chromosomes, and preparations often deteriorate when made permanent.

The writer has recently discovered a more selective stain, namely orcein, another natural dye which can be used with acetic acid.¹ There is no reference in the literature to its having been used as a stain for chromosomes.

Acetic orcein is equally effective with fresh and permanent preparations. The staining is more easily regulated than with carmine and in very few finished preparations is any color retained by the cytoplasm. Coloration of the cytoplasm is a defect often noticeable in aceto-carmine preparations.

¹Apparently it is difficult to obtain standardized samples of orcein free from all impurities. The two samples of dye used during the course of these observations were supplied by British Drug Houses. No difference in results was noted.

Acetic orcein has been used successfully with a number of plant and animal cells, and its selectivity can be seen in preparation of salivary gland chromosomes, where a band and heterochromatin structure is revealed more clearly than with other methods.

The keeping qualities of acetic orcein are good, altho periodic filtering is necessary with the solutions of higher dye-content, which tend to form a scum on the surface. This scum may be an oxidation product due to impurities in the dye.

Staining is good after acetic alcohol fixation, but becomes increasingly more difficult after storage in 70% alcohol. Tissues that have been pre-fixed should therefore be rinsed in 45% acetic acid previous to staining, and material stored in 70% alcohol should be left in acetic acid for one hour or more. No advantage has been found in the use of iron as a mordant.

To judge from preparations three months old, the stain has good keeping qualities. To obtain permanent preparations, however, one must remember that absolute alcohol is a solvent of the dye and one should therefore avoid mounting media (such as Euparal) which contain alcohol.

TECHNIC

The schedule advised, which is similar to that used with acetocarmine, is as follows:

A. PREPARATION OF ACETIC ORCEIN SOLUTION

The standard solution is 1% orcein in 45% acetic acid. It should be made as follows: Dissolve 1 g. of orcein in 45 cc. of hot glacial acetic acid (near boiling); leave till cold and then add 55 cc. of distilled water. Shake well and filter.

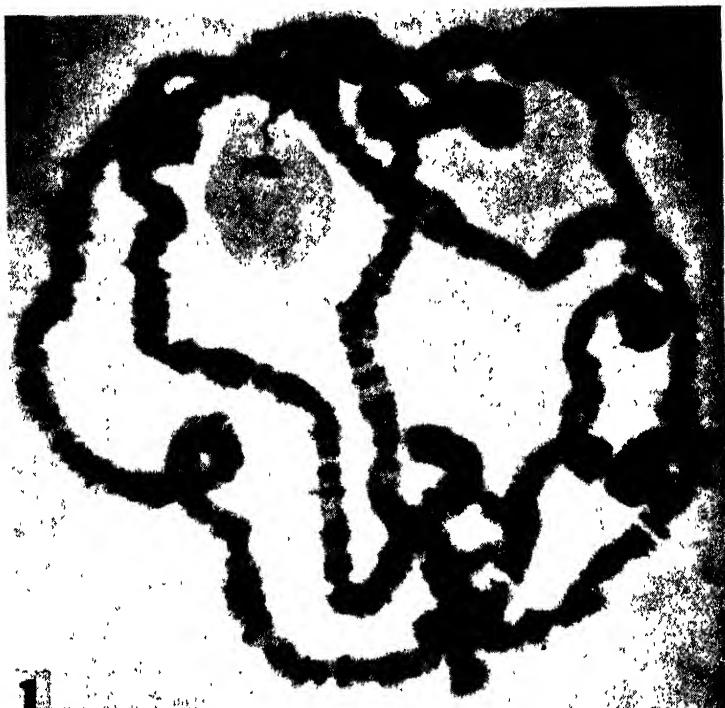
Modified solutions contain up to 70% acetic acid and 0.5-2% orcein.

B. PREPARATION OF TEMPORARY SLIDES (ANTHER OR TESTIS)

1. Tease out cells in a drop of acetic orcein (0.5% orcein is often sufficient for large chromosomes). To secure even penetration, leave 30-60 seconds before placing the cover slip in position. Air bubbles should be avoided.

Note: The following special precaution is worth taking against loss of cells: Smear the cover slip thinly with Mayer's albumen, wipe off excess and then heat carefully over a low flame till a gray smoke is given off. Use when cool. Most of the cells will then remain attached to the cover slip.

2. Pass the slide carefully two or three times over a low flame; do



1

2



Fig. 1. *Drosophila* sp., salivary gland, showing nucleolus and organiser. Standard acetic-orcein solution, followed by acetic-orcein gelatin medium. $\times 900$.

Fig. 2. *Tradescantia bracteata*, pollen mother cells. Half strength orcein. $\times 1,400$.

Fig. 3. *Crocus balansae*, root tip, metaphase. Standard acetic-orcein solution plus N HCl. $\times 1,900$.

not allow to boil. The heating of slides made from stored material needs extra care, for an excess of heat results in complete destaining.

3. If very flat plates are required they are best obtained by applying an even pressure on the cover slip under several thicknesses of blotting paper. The cover slip should not be allowed to move during this operation, and it is best done before heating. With aceto-carmine (perhaps on account of its temporary hardening action) Belling² recommended pressure after two or three days, but since tissues remain relatively soft in acetic orcein, this delay is unnecessary.

4. The slide can now be made permanent, or ringed as a temporary mount with a view to making permanent at a later date. It should, however, be remembered that, as with aceto-carmine, the intensity of the stain in temporary mounts will increase with age. For this reason they are best kept in a cool dark place.

C. MACERATED ROOT TIPS

The addition of 1 cc. N HCl solution to 10 cc. of the standard orcein solution assists in the softening of plant tissues such as root tips, young embryos and ovary walls. The tissues can be stained fresh, or after pre-fixation in acetic alcohol. The suggested method is as follows:

Rinse tissues in 45% acetic acid, after acetic alcohol fixation. Place in watch glass, containing a few drops of the solution, and heat gently 3-4 times over a low flame; do not boil. Slice the tissues into thin sections in a drop of fresh stain on a slide; crush with a scalpel or blunt end of a needle holder. Leave 3-4 minutes before placing the cover slip in position. Squash by blotting under several thicknesses of blotting paper, heat gently over a spirit flame, seal or make permanent.

Preparations that are useful for many purposes can be obtained quickly by this method.

D. SALIVARY GLANDS OF DROSOPHILA

For salivary glands of *Drosophila* 2% orcein was found to be the most suitable. The best preparations of *D. melanogaster* and *D. miranda* were obtained when the acetic acid content was raised to 70%. The following method is advised:

1. Dissect glands in Ringer solution or drop of acetic orcein. Leave in stain 5 to 10 minutes.

²Belling, J. 1930. *The Use of the Microscope*. McGraw-Hill, New York. See p. 242.

2. Dry stain accumulates by evaporation round the rim of the drop; remove this by carefully wiping with moistened blotting paper before placing the slip in position.

3. For flattening plates acetic orcein, on account of its softening effect, requires less pressure than acetic carmine.

E. SALIVARY GLANDS OF SCIARA

The following method was found to be most suitable:³

Dissect out glands in 45% acetic acid and transfer to a drop of the standard acetic orcein solution containing 10% chloroform. Staining for 2-3 minutes is generally sufficient. Altho only a trace of the chloroform diffuses into the acetic acid, the bulk remaining as a layer at the bottom of the vial, this trace has a slight hardening effect on the tissues which enables one to obtain excellent preparations with no color in the background.

F. PERMANENT PREPARATIONS

1. Separate the slide from the cover slip by turning it face down in a ridged dish containing 10% acetic acid; after 3-10 minutes the cover slip will fall off.

2. Pass the slide and cover slip (with salivary glands all the material will remain attached to the cover slip if treated as above with albumen) thru the following solutions: 80% alcohol, 2 minutes; absolute alcohol, 2 minutes; two changes of cedarwood oil, 5 minutes in each (a longer time does no harm).

3. Recombine slide and cover slip by mounting in thick immersion oil, or balsam. Blot carefully to remove excess cedarwood oil, dry on hot plate before cleaning.

USES OF ORCEIN

1. Orcein can be substituted for carmine in Zirkle's gelatin combination³ (none of Zirkle's other mixtures have been experimented with as yet). This orcein-gelatin combination was found to be most useful in showing the structure of the nucleolus and its attachment to the chromocenter in species of *Drosophila*. For this purpose it is recommended to be used after fixing and staining with standard acetic orcein solution, either before placing the cover slip in position, or after separation in 10% acetic acid, depending on the degree of

³Zirkle, C. 1940. Combined fixing, staining and mounting media. *Stain Techn.*, 15, 189-58.

staining required. It can also be used for pollen mother-cells and other tissues. The modified formula is as follows:

| | |
|--------------------------|--------|
| Gelatin..... | 10 g. |
| Sucrose..... | 10 g. |
| Glacial acetic acid..... | 50 cc. |
| Distilled water..... | 60 cc. |
| Orcein..... | 1 g. |

The omission of stains from Zirkle's medium makes it an ideal ringing medium, which dissolves in the 10% acetic acid solution used for separation, should it be wished to make the slides permanent at a later date.

2. Orcein can also be used successfully in mixtures of propionic and acetic acids, or a lactic-acetic combination, consisting of equal parts of glacial acetic acid, lactic acid and distilled water plus 0.5% orcein. Preparations made with this mixture will keep without ringing from 7 to 14 days. The stain is a little slower in acting, but is intense enough after a few hours. The use of lactic acid was suggested by my former colleague, Dr. A. C. Fabergé.

IDENTIFICATION OF SPERMATOZOA IN CRIMINOLOGICAL INVESTIGATIONS

J. D. LAUDERMILK, *Pomona College, Claremont, Cal.*

In certain types of medico-legal examination, it is frequently of great importance to be able to make specific identification of spermatozoa. In the great majority of these cases, it is required that the cells should be isolated from dried material on cloth. Several procedures have been proposed and put into practice to this end. In general, these methods consume considerable time and are relatively complicated. The evidence requires much handling, and, as one of the leading authorities¹ says ". . . From a long experience of this work, we are able to state that it is not so easy a matter as the student may imagine from the ordinary accounts in books. . . ." While relatively simple in theory, it becomes much more complicated in actual practice and sometimes operates to the chagrin of even the professional microscopist who is inexperienced in this particular work.

During the past several years, I have made many examinations of this type for the police and have been constantly aware of the drawbacks mentioned by the above authority. In view of this deficiency the following technic has been developed, which has invariably given satisfactory results. The schedule is applicable to stains at least a year old. In most cases this is a much greater lapse of time than will be required. The schedule follows:

1. Locate the stained areas under ultra-violet light. This is very easily done by fitting a *Corning* filter No. 587² to an ordinary three cell flashlight by means of a simply constructed housing. This filter transmits soft ultra-violet of from 3200 to 3900 Å. Under this illumination, stains resulting from seminal fluid, when dry, show up as sharp, bluish-white, clearly mapped-out areas which are highly fluorescent.

2. Having located the suspected areas, remove pieces of the cloth about 4 mm. square by cutting out with dissecting scissors. Where the suspected area is small, only half the sample should be taken, so that untouched material may remain for further examination if required. Prepare as many slides as there are samples taken.

¹Glaister, John. 1938. Glaister's Medical Jurisprudence and Toxicology. 6th ed. E. & S. Livingstone (16-17 Teviot Place), Edinburgh. See p. 408, 2nd paragraph.

²Corning Glass Co. Corning, N. Y. Heat resisting, red-purple ultra No. 587.

3. In the center of each slide place a small drop of cold water. Shove the cloth square into the drop. If the cloth is simply placed on top of the drop, this is apt to spread and make a slovenly looking smear. Let the cloth soak for one minute.

4. Remove excess water with a piece of filter paper so that no overflow covers the square. The cloth should be wet but there should be as little excess water as possible. Add one drop of *boiling* distilled water and allow the preparation to stand for one minute. Remove excess water and repeat the operation three times.

5. After the third treatment, shred the wet cloth square with the needles until well teased out. The yarns should be well separated.

6. Allow the slide with the teased out yarns to dry on a hot-water plate. This can be extemporized from a plate of galvanized iron placed over an ordinary copper hot-water bath. When the slide is perfectly dry, remove the bulk of the yarns and fibers with needle and forceps. This operation generally requires about five minutes; a smear will remain.

7. Stain the smear for two minutes with a solution of basic fuchsin³ containing 25 mg. of the dye in 10 cc. of water. Apply the stain with a dropper and spread out over the smear with a needle.

8. Wash the slide six times by flooding with distilled water squirted on above the stained smear by means of the dropper while the slide is held in an inclined position over the waste-jar. Dry again on the hot-water plate, mount in balsam and examine. Spermatozoa are deeply stained and show up sharply by this treatment.

After having had experience with practically all the generally used methods for isolation and identification of spermatozoa in cloth, I have found it to be practically useless to examine unstained material or temporary mounts. Where this type of evidence has to be exhibited in court, permanent mounts are absolutely necessary. By the schedule given above, and which may be completed in twenty minutes, I find that the cells are not only hardened so that the tails are preserved in many cases, but that the whole cells are fixed to the slide by the action of the boiling water on the albuminous material extracted from the stained cloth. Handling of the evidence is reduced to a minimum. Results have invariably proved satisfactory with stains on cotton and rayon more than a year old. There is only slight probability that any evidence will be lost in manipulation.

³Basic fuchsin, total dye content 82%. National Aniline & Chemical Co. Inc., New York.

NOTES ON TECHNIC

A PLASTICIZED POLYSTYRENE MOUNTING MEDIUM. Slides mounted in synthetic resin media frequently retract badly under the coverslip in drying, an objection previously noted by Mohr and Wehrle.¹ Kirkpatrick and Lendrum² minimized this difficulty in their trials of synthetic mounting media by incorporating the plasticizer tricresyl phosphate, and found that a British-made polystyrene resin was satisfactory when so plasticized. An American-made polystyrene resin (sample No. XMS 10023) obtained thru the kindness of the Bakelite Corporation appears to be equivalent to that described by the British workers. Mounts set very hard with little retraction when dried at room temperature or in the paraffin oven if a slight excess of the mounting medium is left around the margin of the cover slip. Brain tissue stained with thionin for demonstration of Nissl substance has shown slight fading after one year. Routine pathological tissues stained with hematoxylin and eosin, and with Lillie's modified Masson, in Dr. M. M. Mason's laboratory, have not faded after five months. The older and dryer mounts remain water-white and become unusually clear, probably because of the higher refractive index (1.60-1.62) as compared with damar (1.52).

Preparation: suspend 60 g. of resin in a mixture of 100 ml. of toluene and 18 ml. of tricresyl phosphate, and place the stoppered container overnight in the paraffin oven. By morning the resin will usually have dispersed to form a water-clear syrup which is then decanted for use.

The resin is obtained as clean, dry, white pellets. The price is low: in 1940 approximately \$0.68 per pound if purchased in small lots. The tri-*o*-cresyl phosphate was a gift of the Monsanto Chemical Company. A purified Eastman grade was also satisfactory; no experiments were conducted with the much less expensive technical grade. It should be noted that tricresyl phosphate is a violent poison if ingested.—S. H. HUTNER,³ Middlesex Univ., Waltham, Mass.

AN IMPROVED DILUTION FLUID FOR ERYTHROCYTE COUNTS. The following fluid appears more convenient than Hayem's or Toisson's

¹Mohr, J. L., and Wehrle, Wm. 1940. Resins for sealing glycerin mounts. *Stain Techn.*, **15**, 174-5.

²Kirkpatrick, J., and Lendrum, A. C. 1939. A mounting medium for microscopical preparations giving good preservation of colour. *J. Path. & Bact.*, **49**, 592-4.

³Present address: 342 Commonwealth Ave., Boston, Mass.

for red cell counts: iodine, 0.3%; potassium iodide, 0.4%; sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$), 1%. It is prepared as follows: dissolve the potassium iodide in 50 ml. of distilled water; add the iodine crystals, using gentle heat to accelerate their solution; then add the citrate, make up to 100 ml. and filter. With this fluid the erythrocytes are stained brown against a clear background, while leucocytes are destroyed. The iodine stain is easily removed from the walls of the counting chamber with the usual glassware-drying solvents. The erythrocytes are not altered in morphology or clumped even at the end of an hour's standing in the counting chamber. Exposure of the solution to direct sunlight over a period of ten days did not result in appreciable change.—LUIS A. VALLARINO, Department of Bacteriology and Immunology, Middlesex Univ., Waltham, Mass.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

COLE, ELBERT C. *Textbook of Comparative Histology.* 6 x 9 in. 396 pp. Cloth. 297 illustrations. The Blakiston Company, Philadelphia, Pa. 1941. \$4.00.

Altho not so implied in the title, this book actually deals with animal histology only. Most of the book is a discussion, profusely illustrated with fine photomicrographs, of the histology of animal tissue and of the tissue-complexes making up the various systems of organs. The last three chapters, however, (65 pp.) deal with instruments and methods, thus bringing the book within the scope of these reviews. These three chapters deal respectively with: "The Use and Care of the Microscope"; "Methods of Recording Data"; "Histological Technique". The last of these chapters treats a large subject in a very brief manner; it appears more useful for reference purposes than as a guide to be followed by a laboratory worker.

MICROSCOPE AND OTHER APPARATUS

HERTZMAN, ALRICK B. A simple, inexpensive, vertical illuminator for the student microscope. *J. Lab. & Clin. Med.*, 26, 868. 1941.

There is need for vertical illumination for examining human skin capillaries and minute vessels in the frog. The following method is simple and efficient: Mount a pencil-flash-light bulb, Mazda 222, (operating on 2.2 v. current) in a screw type dial socket carried by a copper wire 10 in. in length, and $\frac{1}{8}$ to $\frac{1}{4}$ in. in diameter. Clamp this to a ring stand. As a source of current, use dry cells or a filament transformer operating at 2.5 v. which may be reduced to 2.2 v. by a resistance wire in the lead to the bulb. The 2.5 v. current may be used to provide a brighter light for photomicrography.—John T. Myers.

JOHN, K. Über ein neuartiges Integriergerät und seine Anwendung insbesondere in der Biologie. *Zts. wiss. Mikr.*, 57, 163-8. 1940.

R. Fuess of Berlin has just put on the market an integrating device permitting direct differential area determinations under the microscope. It consists of a special mechanical stage coupled by a flexible shaft to a very compact, motor-driven, counting apparatus. The latter is equipped with seven push buttons, each placed adjacent to a cyclometer dial. A special grating is provided for the eye-piece. The instrument is operated by moving the mechanical stage in a horizontal direction following the guide lines in the eye-piece. When desired to estimate the area of various cell types, a push-button is arbitrarily assigned to each type and depressed while moving the object along the guide line. Vacant spaces and artifacts to be discounted are estimated on a special push-button, making it possible to estimate the comparative areas of various cell types in a given field. Volumetric measurements may readily be made by multiplying the areas obtained by the thickness of the section.—J. M. Thuringer.

KAUSCHE, G. A. Ergebnisse und Probleme der experimentellen Virusforschung bei Pflanzen (mit übermikroskopischen Aufnahmen). *Ber deut. botan. Ges.*, 58, 200-22. 1940.

The writer discusses the value of the electron microscope in the study of plant viruses. He points out that at a magnification of 16,000 diameters the so-called Stanley crystals appear to consist of bundles of smaller units arranged parallel to

the long axis. These smaller units have dimensions within the range demanded of virus molecules. The reaction of the virus with colloidal gold is shown in photographs. At the neutral point no reaction occurred. The addition of NaCl caused flocculation, while a gold sol acidified with HCl showed the gold particles laid down singly in the virus molecule or its aggregate. When an alkali is added, dispersion takes place due to the disintegration of the virus.—*Merritt N. Pope.*

SCHNEIERSON, S. STANLEY. A simple, clean, cedar oil bottle. *J. Lab. & Clin. Med.*, 26, 1847. 1941.

The following bottle for immersion oil is clean and convenient: Take the dropper from an ordinary staining bottle with ground glass joint, and draw out the tip to a fine point, using the pilot light of a Bunsen burner. Cut off the tip leaving a very small opening of such a size that oil will not drop out by gravity. Fill the hollow part of the dropper with a Pasteur pipette and apply the rubber bulb. A controlled amount of oil can be dropped on a slide. No smearing, evaporation, or hardening of the oil will occur.—*John T. Myers.*

MICROTECHNIC IN GENERAL

GROAT, R. A. Two new mounting media superior to Canada balsam and gum damar. *Anat. Record*, 74, 1-6. 1939.

Two new synthetic resins, Nevillite V and Nevillite No. 1, cycloparaffines or naphthene, are found to be superior to the natural resins Singapore damar No. 1 and Canada balsam as mounting media for histological sections. Nevillite V and No. 1 are superior to the natural resins in that they are homogeneous, clear, transparent, water-white, pure, neutral, have a higher melting point (which is desirable for micro-projection), a slightly higher refractive index, and are less expensive. The color of Nevillite V is more stable than that of Nevillite No. 1 and its refractive index is a little lower; otherwise they are of equal value. A 60% solution of the Nevillites in 40% toluene by weight is recommended.—*W. R. Hunt.*

DYES AND THEIR BIOLOGICAL USES

ALBERT, ADRIAN. Acridine compounds in peace and war. *Chem. and Ind.*, 60, 253. 1941.

Some of the acridine derivatives are of great value in surgery because they kill disease germs without injuring human leucocytes. Of the common amino-acridines, namely acriflavine, euflavine and proflavine, the last has been most strongly recommended as an antiseptic by recent workers. It has been found that 2:7-diaminoacridine is a most promising antiseptic for application to the living brain in head injuries so common in war time. Because of the fluorescence of acridine compounds they are used in titrating opaque or highly colored solutions under an ultra-violet lamp. Radley and Grant include many acridine compounds in their list of standard indicators. Many other acridine drugs are used in human amoebiasis, malaria, tabes and lambliasis, as well as in mammitis, and other infections of cattle. Another spectacular property of certain acridine compounds is their ability to chemiluminesce or give off "cold light". Further development in this interesting research is necessary, especially on the methods of synthesis which are now tedious and unsuited to large scale manufacture.—*A. P. Bradshaw.*

FERREBEE, J. W., LEIGH, O. C. and BERLINER, R. W. Passage of the blue dye T-1824 from the blood stream into the lymph. *Proc. Soc. Exp. Biol. & Med.*, 46, 549-53. 1941.

The injection of blue dye, T-1824, into the jugular vein of dogs was followed by the appearance of approximately 20 to 40% of the plasma concentration of dye in the thoracic lymph after 2 hr., and about 8 to 15% in the cervical lymph. It is estimated that about 7.5% of the injected dye passed thru the thoracic duct in 2 hr. The studies are pertinent to the knowledge of blood plasma volume in connection with shock.—*M. S. Marshall.*

ANIMAL MICROTECHNIC

BAKER, J. R. Chlorazol black E as a vital dye. *Nature*, 147, 744. 1941.

Chlorazol black E (C.I. 581) possesses unusual ability to be taken up quickly and completely by the reticulo-endothelial system. A 1% solution in distilled water is convenient for subcutaneous injection into mice. The dye is non-toxic and is not excreted. Vitaly stained preparations may be counterstained with safranin and orange G after Zenker fixation.—*S. H. Hutner*.

COPLEY, ALFRED L., and ROBB, TOM P. A new direct method of counting the blood platelets. *Amer. J. Physiol.*, 133, 248. 1941.

One cc. of venous blood is drawn into a syringe containing 1 cc. of modified Aynaud solution (NaCl 0.75%, sodium citrate 3.8%, formaldehyde 3.7%). After mixing, the fluid is stored in a clean, dry, stoppered test tube. When a count is to be made the tube is inverted 10 times and 5 cc. of the mixture is added to 12 cc. of 3.8% sodium citrate. This second dilution is mixed and 0.5 cc. of it is added to 0.5 cc. of 0.2% brilliant cresyl blue in 3.8% sodium citrate. The final mixture is placed in a counting chamber. The color of the red blood cells fades after 10 min., and the platelets appear as dark blue bodies.—*Elbert C. Cole*.

DE BOISSEZON, P. Une modification de la technique de coloration de la myéline au noir Soudane B. *Bull. d'Histol. Appl.*, 18, 90. 1941.

The technic described by Lison and Dagnelie (*Bull. d'Histol. Appl.*, 12, no. 2, 1935) for staining myelin is unsatisfactory for study of the peripheral nervous system because of the dark preparations resulting from the dark color of the fat of the conjunctive tissue. The following modification has been developed to avoid this difficulty: Place the frozen sections in acetone for 15 min. (this dissolves the neutral fats leaving the myelin intact); 70% alcohol, 10 min.; Sudan black B dissolved in 70% alcohol and filtered, 5 min.; differentiate in 60% alcohol; wash in distilled water; mount in glycerin gelatin. This gives preparations in which the myelin nerves and their endings appear dark blue with a clear background.—*Jean E. Conn*.

GOMORI, G. Observations with differential stains on human islets of Langerhans. *Amer. J. Path.*, 17, 395-406. 1941.

A study of the differential staining of α , β and δ cells of human pancreas (within 4 hr. after death) led to improvement of the chromium-hematoxylin-phloxine stain. The following procedure was used: Fix 2 min. slices in Bouin's 30 min., then halve with a razor to 1 mm., and continue to fix for 24 hr. Prepare paraffin sections on slides and run down to water in the usual manner. Refix in Bouin's, 12-24 hr. Wash thoroly in tap water to remove picric acid. Treat 1 min. in an aqueous solution of 0.3% each of $KMnO_4$ and H_2SO_4 . Decolorize with 2-5% aqueous sodium bisulfite and wash. Prepare the hematoxylin solution by mixing equal parts of a 1% aqueous solution of hematoxylin (National Aniline, C. P., or Grubler's dark) and 3% chromium alum; then, to each 100 cc. of the mixture, add 2 cc. of 5% $K_2Cr_2O_7$ and 2 cc. of 0.5 N H_2SO_4 . Let ripen 48 hr. and filter. Stain under microscopic control until the β cells stand out deep blue (10-15 min.). Differentiate in 1% HCl-alcohol for about 1 min. Wash in tap water until the sections become a clear blue. Counterstain with a 0.5% aqueous solution of phloxine (source not stated) for 5 min. Rinse and transfer to 5% phosphotungstic acid solution for 1 min. Wash in tap water for 5 min., in which treatment the red color should reappear. Differentiate in 95% alcohol; but, if the section is too red and the α cells do not stand out clearly enough, rinse 15-20 sec. in 80% alcohol. Transfer to absolute alcohol, clear in xylene and mount in balsam. Sixteen figures which show the author's stain and also results with the Mallory-Heidenhain azan stain and Mallory's phosphotungstic acid hematoxylin are included, together with studies of the pancreatic islets under normal and pathological conditions.—*H. A. Davenport*.

LOWRY, OUIDA, BEAMS, F. H. W., and KING, R. L. The fibrillae of the uterine cells of *Ascaris equorum* (variety *univalens*). *J. Morphol. & Physiol.*, **68**, 585-91. 1941.

Pieces of the uteri of living worms were fixed in several fixing solutions, of which alcohol-acetic (9 parts of 70% alcohol and one part glacial acetic acid) was best. Tissues were embedded in paraffin and cut from 4 to 10 μ thick. Of several stains tried, Heidenhain's hematoxylin (following alcohol-acetic fixation) gave best results. Both basement membrane and fibers stained bluish black.—*Elbert C. Cole.*

MONKE, J. VICTOR. A method for counting the leucocytes in blood containing gum acacia. *J. Lab. & Clin. Med.*, **26**, 1664-7. 1941.

After the injection of gum acacia into the blood stream, it is not possible to obtain satisfactory leucocyte counts using Turck's Solution as the lytic diluent. The following dilution solution is recommended: Saponin, 0.1%, and methyl violet 0.1% in 0.85% saline. This solution produces complete hemolysis and stromolysis. The white blood cells are easily counted.—*John T. Myers.*

PAPANICOLAOU, GEORGE N. Some improved methods for staining vaginal smears. *J. Lab. & Clin. Med.*, **26**, 1200-5. 1941.

The following complete method is satisfactory for both human and animal vaginal smears: Fix the moist smear in a mixture of equal parts alcohol and ether, rinse in 70% alcohol, then 50% alcohol, then water. Place in Ehrlich's hematoxylin (or other hematoxylin) for 2 min. Rinse in distilled water, keep in running water for 5 min. or in Li₂CO₃ (8 drops concentrated aqueous solution in 100 cc.—exact concentration not stated) for 1 min. Rinse in distilled water. Stain in a counterstain such as the following for 2-5 min.: aniline blue W.S. (0.5% aqueous solution) 12 cc., orange G (0.5% aqueous solution) 25 cc., acid fuchsin (0.5% aqueous solution) 21 cc., eosin Y (0.5% aqueous solution) 42 cc., phosphotungstic acid 0.1125 g., phosphomolybdic acid 0.225 g. Rinse in water. Rinse in dioxan 10 to 15 times until clear. The staining time may be shortened by omitting the hematoxylin. Total cornified cells stain with the yellow dye. Smears must not be permitted to dry because the staining reaction of the cells will be changed.—*John T. Myers.*

TUREVICH, E. I. Nucleal reaction of the elementary bodies. *Bull. de Biologie et de Médecine Expérimentale de l'U.R.S.S.*, **9**, 222-4. 1940.

If nuclei stained violet by the Feulgen reaction are viewed by dark-field with carbon arc illumination, they show a bright yellowish-green luminescence (fluorescence ?); control nuclei not subjected to preliminary acid hydrolysis (*N* HCl for 6 min. at 60°C., then 1 min. in the cold solution), and which therefore do not stain, do not show this dark-field luminescence. The elementary bodies of infectious ectromelia, chicken pox and vaccinia, after treatment by the Feulgen procedure, are invisible by bright field, but display a green luminescence by dark-field similar to that observed with the nuclei stained for controls. The accompanying bacteria have a violet hue by bright field, and pronounced green luminescence by dark-field. It is concluded that the apparent negative Feulgen reaction of elementary bodies follows from the small size of the virus particles rather than from a lack of nuclear material.—*S. H. Hutner.*

PLANT MICROMECHANIC

JOHANSEN, D. A. A quintuple stain combination and an evaluation of dyes suitable for multiple staining. *El Palo Alto News*, **6**, 1-4. 1941.

Exceptionally good differentiation of plant materials has been secured by the use of a combination of five dyes. Among the materials used to test the differentiation were mature embryos of corn, stems of *Aristolochia*, microsporocytes and megagametophytes of lily and thalli of *Nemalion*. Large numbers of slides may be stained in a rack by the following method: Pass the slides thru xylol down to 70% ethyl alcohol and then into a safranin solution (dissolve 4 g. safranin 0, NMv-6, in 200 cc. methyl cellosolve, add 100 cc. each 95% alcohol and distilled water, then add 4 g. sodium acetate and 8 cc. neutral formalin) for 24 hr. or more.

Rinse in running water 10 sec. Stain in a 1% solution of methyl violet NE (C.I. 680), in distilled water for 5 min. Rinse in running water 10 sec., then in equal parts methyl cellosolve, 95% ethyl or pure isopropyl alcohol, and tertiary butyl alcohol for 10 sec. Stain 5 min. in the green dye (dissolve completely 1 g. fast green FCF, LGf-1, in 200 cc. methyl cellosolve, add 1 g. malachite green, LMg-3, C.I. 657, shake thoroly, and, when dissolved, add 100 cc. each 95% ethyl alcohol and tertiary butyl alcohol, acidify with glacial acetic acid to make about 1%). Rinse in 0.5–0.8% glacial acetic acid solution in water for 10 sec. Rinse in cellosolve alcohol mixture as used in previous step acidified with glacial acetic acid to 0.5%, 10 sec. Stain about 5 min. in an orange solution (a saturated solution of orange I, C.I. 150, or orange II, C.I. 151, in methyl cellosolve; to this add 100 cc. each clove oil, 95% ethyl alcohol, and tertiary butyl alcohol). Rinse 10 sec. in equal parts methyl cellosolve, absolute ethyl or isopropyl alcohol, tertiary butyl alcohol, clove oil, and terpineol (not terpinol). Rinse 10 sec. in equal parts xylol, absolute ethyl or isopropyl alcohol, methyl cellosolve, tertiary butyl alcohol, and terpineol. Rinse 10 sec. in xylol plus 10% absolute ethyl alcohol. Rinse 10 sec. in each of two changes of xylol. Mount in balsam dissolved in benzol.

Other dyes were suggested for various special purposes. The dyes were from National Aniline or Hartman-Leddon; and the technic has been standardized for the particular batches mentioned. This makes it a difficult procedure to follow; this is especially true since the designation NMv-6, given for Safranin O, happens to be a certification number applying to a batch of methyl violet.—*Virgene Kavanagh.*

KISSEK, J. Versuche über Trockenkonservierung von Zweigen und Knospen in natürlicher Form und Farbe. Ber. deut. botan. Ges., 58, 256–68. 1940.

The natural form in twigs and buds may be preserved by laying them in water until they have swollen completely, then transferring to 40–50% glycerin until thoroly saturated. If the material has been killed and fixed, it is especially important to get complete penetration of the glycerin solution. Since long soaking is harmful to the color and produces abnormal bendings of the buds, repeated evacuations of the immersed twigs with the filter pump, followed by leaving several days in the evacuated condition, are advisable. If the pieces are large or the buds impervious, it will be necessary to pierce the tissue with a fine needle. When infiltration is complete, the material is brought out of the solution, dried superficially and laid in an airy place until the water has evaporated.

The green color can be preserved by the use of copper salts (10% CuSO₄, 7% Cu(CHO₂)₂ or 5% CuCl₂) in 10% acetic acid. Should the resulting color be too bluish, a salt of zinc (1 part copper to 1 to 5 parts zinc) or of uranium (1 part uranium to 5–10 parts copper) may be substituted for part of the copper salt.

The brown color is seldom affected by the agents used in preserving the green. When disturbing colorations appear, a treatment with a solution of SO₂ is possible without damage.

The preservation of the red or anthocyanin colors is still not possible in many cases, but in twigs and buds it is best obtained by means of formol in the solution.

A fluid recommended for twigs and buds with exceptionally brown, or brown and red colors is 25 cc. formol and 75 cc. of 60% glycerin. Where there is only green, or green with red or brown, a mixture of 25 cc. formol, 10 cc. acetic acid, 50 cc. glycerin and 15 cc. H₂O is preferred. To the latter mixture should be added the desired amounts of powdered salts of copper or copper with zinc or uranium.—*Merritt N. Pope.*

SCHAEDE, R. Über den Feinbau von Parenchymmembranen. Ber. deut. botan. Ges., 58, 275–90. 1940.

For investigating the fine design structure of the parenchyma membrane in the pith and root tissue of various plants, the chlorzinciodide of Van Wisselingh was used. It is prepared by dissolving 15 g. ZnCl₂ in 10 cc. distilled water and adding 0.5 g. KI and 0.25 g. iodine. The iodine is in excess and insures a concentrated solution. After about 2 days at room temperature, the solution takes on an amber color and is ready for use, giving an intense, pure, dark blue stain. If the preparation is ringed, it will hold its color for about a month.—*Merritt N. Pope.*

SCHUSTER, C. E. Method for softening filbert buds imbedded in paraffin. *Botan. Gazette*, 102, 815-7. 1941.

The following procedure, altho possibly unsuited for the study of fine details, is recommended for gross studies of pollen tubes, tube cysts, and similar developments in the pistil of hard buds like those of the filbert:

Killing and fixing fluid. Solution A: 2% aqueous chromic acid, 10 cc.; 95% ethyl alcohol, 100 cc.; 86% acetic acid, 100 cc. Solution B: 40% formalin, 40 cc.; 95% ethyl alcohol, 100 cc. Mix solutions A and B in equal parts.

Procedure. Dissect out those parts of the buds needed for study and immerse in this fluid for at least 24 hours. Boil in 50% dioxan in a water bath under a ventilating hood for 40 min. Pour off dioxan and run thru several baths of ZnCl in increasing strength, prepared as follows: Dissolve 10 g. ZnCl in 35% HCl, and dilute with distilled water, in the following volumetric proportions for each of the first four baths: 1:7; 1:9; 1:1; 3:1. The fifth bath is undiluted. Treat 2 hr. in each of the first three baths, 1 hr. in the fourth and 15-30 min. in the fifth according to the degree of hydrolysis desired. Then dehydrate in three changes of 25% dioxan, and one each of 50% and 75%, allowing 2 hr. in the first strength and 1 hr. in each of the others; then 3 half-hour changes of 100% dioxan mixed with equal parts of butyl alcohol, adding 1 drop of oil (kind unspecified) to each cubic centimeter of the last change.

Put in vials with shaved paraffin, place on top of paraffin oven overnight, and in oven later for 3 or 4 half-hour changes of pure paraffin. Do not keep in oven longer than necessary, to avoid hardening the hairs.

Cut embedded material into blocks $\frac{1}{3}$ - $\frac{1}{2}$ inch square and soak in water for several months (e.g. 6 months in the case of filbert buds). Place for 2 weeks or more in open dishes containing a bacterial infusion prepared by decaying straw and grass in 0.05% aqueous NH_4NO_3 ; keep in a warm room out of sunlight, and as evaporation progresses add more of the NH_4NO_3 solution. Neither of these two steps alone produce sufficient softening for good sectioning.

After the softening, whole buds can be sectioned at 10μ , the pistils and attached tissue at $5-7\mu$. During sectioning the paraffin blocks (previously chilled in an ice-bath) are cooled from time to time with pieces of ice. The knife should be specially sharpened according to the directions of Evenden and Schuster.

—K. A. Stiles.

MICROÖRGANISMS

FIELD, J. W. A new rapid method of staining malarial parasites in thick blood films. *Bull. from Inst. Med. Research (Federated Malay States)*, No. 2 of 1941.

Solutions of methylene blue (containing also methylene azure) and of eosin, made isotonic with blood plasma, both buffered to pH 6.6, are used to provide a new rapid method for staining thick blood films. The use of isotonic solutions avoids the osmotic stresses to which Giemsa-stained thick films are exposed. The "finger-slide" and "pipette" methods are recommended for making the films, which may be stained within a few minutes of preparation (as soon as they cease to be obviously moist). Fixation is unnecessary. The method of staining is as follows: Dip the film for 1 or 2 sec. into stain A (methylene blue, 0.8 g.; azure I, 0.5 g.; anhydrous Na_2HPO_4 , 5.0 g.; anhydrous KH_2PO_4 , 6.25 g.; distilled water, 500 cc.). Rinse 5 sec. in clean water. Dip for 1 or 2 sec. into stain B (eosin, 1.0 g.; anhydrous Na_2HPO_4 , 5.0 g.; anhydrous KH_2PO_4 , 6.25 g.; distilled water, 500 cc.). Rinse for 2 or 3 sec. in clean water. The solutions are prepared by dissolving the phosphates first, then adding the stain, and filtering after 24 hr. (The writer used eosin from the British Drug Houses and the other two dyes from Gurr.) The slides are placed vertically to dry; the best blood picture is obtained at the lower edge of the film.

The advantages claimed for this method are shortening of the preliminary drying of the blood, reduction of the time of staining, better preservation of the form of leucocytes and blood protozoa, and maintenance of correct pH by buffering. The only serious drawback is that the stain is liable to give confusing appearances in anemic blood; the chromatoid and reticular residues of immature red cells stain more obtrusively than with Giemsa's stain and may confuse an inexperienced worker.—Jean E. Conn.

GRAY, P. H. H. Staining of bacteria and certain fungi. *Nature*, 147, 329. 1941.

Spores and vegetative cells may be stained differentially in one operation by the following method: stain fixed films of bacteria or yeasts for 1 min. over steam with a solution of 0.5% malachite green and 0.05% basic fuchsin in distilled water; wash and dry. Spores stain greenish-blue, vegetative cells violet or pink. Acid fast bacteria retain the greenish-blue tinge, sometimes showing violet granules. A saline dilution of the staining solution has been developed for differentiating the hyphae and conidia of certain fungi in agar plate cultures. More details are to appear in a later publication.—*Jean E. Conn.*

HORVATH, J.v. Die Anwendung der Karminessigsäure für die Kernfärbung bei den Ziliaten. *Zts. wiss. Mikr.*, 57, 168-71. 1940.

The author uses the aceto-carmine method for nuclear staining of ciliates of the Hypotrichiae, Peritrichiae, and Holotrichiae. The stain is prepared as follows: glacial acetic acid 45 ml., distilled water 50 ml., add excess of powdered carmine and gently boil from $\frac{1}{2}$ to 1 hr.; when cool, filter and make up to original volume; preserve in a glass-stoppered bottle. The routine technic is varied as follows:

(a) If material is abundant, use the centrifuge method (Gelei, 1934), employing equal quantities of culture and staining fluid and applying 15-20 min.; put on slide and place cover glass over it. For permanent preparations, add 10% glycerin to the staining fluid before centrifuging; and after decanting, replace the discarded fluid with more of the same glycerin-aceto-carmine solution before transferring to a slide.

(b) For individual specimens, spread the material evenly on albumenized slides, fix and stain with aceto-carmine. A small drop of glycerin and cover glass complete the preparation.

For many organisms (e.g. cultures of Peritrichiae in straw infusion or in horse manure) special fixation may be necessary since acetic acid does not fix completely; in this case, 50% glacial acetic acid in absolute alcohol is used with the culture medium in a relationship of 1:1; fixation 5 to 15 min. After fixing the following procedure is recommended: Centrifugalize, pour off fixative, and without washing, add aceto-carmine, staining with occasional shaking 1 to 24 hr. After staining differentiate by placing the test tubes into a water bath from 60°-80° C. for 3-4 min. Agitate tubes frequently. If differentiation is insufficient, add 2-3 drops of glacial acetic acid per each 0.5 ml. stain (with material) and repeat differentiation in water bath at 80° C. If over-differentiation has taken place, pour off the old staining mixture and add a fresh mixture, allowing the material to stand for 5 min. at room temperature.—*J. M. Thuringer.*

PIEKARSKI, G. Über kernähnliche Structuren bei *Bacillus mycoides* Flügge. *Arch. Mikrob.*, 11, 406-31. 1940.

Of 11 fixing agents tested with the Feulgen reaction, the best was concentrated $HgCl_2$ in water. Poor fixation resulted from the use of methyl alcohol, formalin and chromic acid. Consistently good results were obtained with no fixing agent. Staining was as follows: Hydrolyze in *N* HCl 8-10 min. (25-35 min. for spores) at 58°-60° C., rinse in distilled water and stain for at least 1½ hr. in fuchsin-sulphurous-acid (stain spores up to 24 hr.), place in an aqueous solution of SO_2 , for 10 min. and rinse briefly in tap water. Use alizarin-viridian in alcoholic solution as a plasma counterstain.—*Merritt N. Pope.*

WEISS, EMIL. A modification of the Gram stain. *J. Lab. & Clin. Med.*, 26, 1519-21. 1941.

The following technic is recommended: Prepare thin uniform smears. Cover with a solution of 3% gentian violet in 20% alcohol for 3-5 min. Wash with warm water. Cover for 3-5 min. with Gram's iodine of 20 times the usual concentration. Cover with acetone and wash off immediately with water. Counterstain briefly with fuchsin (2% of basic fuchsin in 95% alcohol). The technic is simple, the reagents keep well and the distinction between Gram negative and Gram positive bacteria is good.—*John T. Myers.*

HISTOCHEMISTRY

BARBER, H. N., and PRICE, J. R. Nature of the Feulgen reaction with nucleic acid. *Nature*, 146, 885. 1940.

In checking Semmens' suggestion that the Feulgen reaction is due to purine components of nucleic acids, these authors find that the effect of the piperidine and pyridine is not chemically equivalent to the Feulgen (Schiff) reaction, but is simply due to their basicity, the effect of which was pointed out by Feulgen. Tests of three of the purines used by Semmens gave negative results. It is concluded that the Feulgen reaction is specific for the potential aldehyde groups of chromatin.—*M. W. Jennison*.

BARNETT, S. A., and BOURNE, G. Use of silver nitrate for the histochemical demonstration of ascorbic acid. *Nature*, 147, 542-3. 1941.

The specificity of the test for ascorbic acid by reduction of an acid solution of silver nitrate may be increased by treatment of the specimen with 5% ammonia after the silver nitrate. Some tissues such as developing bone may show non-specific deposition of silver otherwise.—*S. H. Hutner*.

SEMMENS, C. S. Nature of the Feulgen reaction with nucleic acid. *Nature*, 146, 180-1. 1940.

This modified Schiff's reaction is commonly used as a selective chromatin stain. It is generally stated that the color reaction is due to the aldehyde group of the aldose in hydrolyzed nucleoprotein. The author found that such heterocyclic compounds as pyridine and piperidine also restored the original color of the fuchsin. Caffeine, theobromine, adenine and guanine gave positive results in a few hours. It is suggested that the Feulgen reaction with chromatin may therefore be due to the purine components of the nucleic acids.—*M. W. Jennison*.

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